Post-translational modifications regulating Exonuclease 1 in response to replication forks stalling and double-strand breaks

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DEDICATION

- to my family -

SUMMARY

Double-strand breaks (DSBs), which are among the most dangerous DNA lesions, are estimated to occur at a rate of ten per cell per day in primary human or mouse fibroblasts (Lieber, 2010). These naturally occurring DSBs are generated upon collapse of stalled DNA replication forks, replication across nicks, reactive oxygen species of endogenous origin or the untimely action of DNA endonucleases (topoisomerases or RAG and AID) (Lieber, 2010).

Failure to repair damaged DNA has a well-established role in the onset of cancer. Despite the intense effort currently put to the identification of proteins and pathways involved in the recognition of the various forms of DNA damage, we still lack a clear understanding at the molecular level of DNA repair mechanisms and their regulation. In particular, the hierarchy and mutual influence of post-translational modifications (PTMs) on recruitment, function and stability of DNA repair proteins at sites of damage represent new challenges in the field. Appreciating the importance of PTMs will not only allow understanding how dysfunctions of these machineries contribute to the development of cancer and to acquired resistance to therapy, but will also provide the necessary knowledge to target key components of DNA repair pathways and their regulators in the treatment of cancer.

This study is aimed to answer important unresolved biological questions regarding the molecular mechanism that controls the function of Exonuclease-1 (EXO1), a common component of machineries processing stalled replication forks, DSBs and DNA base mismatches.

Previous work from our laboratory demonstrated that the function of human and yeast EXO1 at DSBs and stalled forks, respectively, is rigorously controlled by specific protein-protein interactions (Eid et al., 2010; Engels et al., 2011). Additional studies from our laboratory showed that, in response to stalled DNA replication, the cellular level of human EXO1 is regulated by phosphorylation-dependent ubiquitylation that channels EXO1 to proteasome-mediated degradation (El-Shemerly et al., 2008; El-Shemerly et al., 2005).

In this study we extend these findings and, taking advantage of a combination of molecular biology and biochemical techniques as well as cell biology

assays, we provide new mechanistic insights on the regulation of EXO1. Indeed, by taking advantage of an immunofluorescence-based high-throughput screen followed by image and computational analysis of the acquired data, we identified UBC9-dependent pathways as major effectors of EXO1 stability, indicating that the proteasome-mediated degradation of EXO1 occurring in response to stalled DNA replication is sumoylation-dependent. Moreover we found that the UBC9-PIAS1/PIAS4 pathway controls EXO1 protein stability in vivo both in basal and DNA damaged conditions and we were able to reconstituted EXO1 sumoylation in vitro with purified recombinant human or yeast EXO1 as substrates and components of the sumoylation machinery. The de-sumoylating enzyme SENP6 was found to constitutively interact with EXO1 both in vivo and in vitro and depletion of SENP6 promotes EXO1 degradation. We also showed that sumoylation and ubiquitylation occur sequentially on EXO1 since interfering with the former, by UBC9 depletion or chemical inhibition of the E1-SUMO activating enzyme, compromises the latter. In a second moment, we demonstrated that sumoylation is required for EXO1 recruitment to DNA in response to damage since UBC9 depletion decreases the ratio of chromatin-bound to free EXO1 and the localization of EXO1 at sites of damage, thus resulting in decreased EXO1-mediated resection of DNA ends. In vitro studies combined with mass spectrometric analysis allowed identification of lysine residues K_{655} and $K_{801/802}$ as major sumoylation sites in EXO1; Chromosomes spreads analysis from cells expressing high levels of wild-type EXO1 showed a high rate of chromosomes breaks upon camptothecin treatment. This was not the case for cells expressing a SUMO-deficient EXO1 mutant, pointing to an important mechanism that cancer cells with up-regulated EXO1 gene expression may put in place to survive.

ZUSAMMENFASSUNG

Doppelstrangbrüche (DSB), die zu den gefährlichsten DNS Läsionen gehören, treten Schätzungen zufolge mit einer Rate von etwa zehn pro Zelle pro Tag in primären humanen oder murinen Fibroblasten auf (Lieber, 2010). Diese natürlich vorkommenden DSB entstehen durch den Kollaps von ins Stocken geratenen DNS Replikationsgabeln, Replikation über eine Diskontinuität (DNA nick) hinweg, reaktive Sauerstoffspezies endogenen Ursprungs oder durch die vorzeitige Aktion von DNS Endonukleasen (Topoisomerasen oder RAG und AID) (Lieber, 2010).

Versäumnisse in der Reparatur beschädigter DNS kommen eine gut etablierte Rolle in der Entstehung von Krebs zu.

Trotz der grossen Bemühungen hinsichtlich der Identifikation von Proteinen und Signalwegen, welche zur Erkennung unterschiedlicher Formen von DNS Schädigungen beitragen, fehlt uns bis anhin ein klares Verständnis auf der molekularen Ebene der DNS-Reparaturmechanismen und deren Regulation.

Im Speziellen gelten die Hierarchie und die gegenseitige Beeinflussung von post-translationalen Modifikationen (PTM) in der Rekrutierung, Funktion und Stabilität von DNS-Reparaturproteinen an Stellen von Beschädigungen als neue Herausforderungen auf diesem Gebiet.

Das Erkennen der Wichtigkeit von PTM wird uns nicht nur erlauben zu verstehen, wie Fehlfunktionen dieser Maschinerien zu der Entwicklung von Krebs und dem Aufkommen von Therapieresistenzen beitragen sondern es wird uns auch das nötige Wissen vermitteln um gezielt Schlüsselkomponenten der DNS-Reparaturwege und deren Regulatoren in der Krebstherapie zu nutzen.

Diese Studie zielt darauf ab, wichtige biologische Fragen zu beantworten, die den molekularen Mechanismus betreffen, welcher die Funktion der Exonuklease-1 (EXO1), einer gemeinsamen Komponente der Maschinerien, die ins Stocken geratene Replikationsgabeln, DSB und Basenfehlpaarungen bearbeiten, kontrolliert.

In früheren Arbeiten unseres Labors demonstrierten wir, dass die Funktion von humaner und Hefe-EXO1 bei DSB respektive bei blockierten Gabeln rigoros von spezifischen Protein-Protein-Interaktionen kontrolliert wird (Eid et

al., 2010; Engels et al., 2011). Weitere Studien unseres Labors zeigten, dass in Antwort auf eine stockende DNS-Replikation das zelluläre Niveau von humaner EXO1 von phosphorylationsabhängiger Ubiquitinierung reguliert wird, welche EXO1 dem Proteasom-vermittelten Abbau zuführt (El-Shemerly et al., 2008; El-Shemerly et al., 2005).

In dieser Studie haben wir diese Beobachtungen ausgeweitet und liefern unter Zuhilfenahme von einer Kombination aus molekularbiologischen und biochemischen Techniken sowie zellbiologischen Analysen neue Erkenntnisse über die Regulation von EXO1.

Tatsächlich konnten wir durch ein auf Immunofluoreszenz basierendes High-Throughput-Screening, gefolgt von bild- und computergestützten Analysen der Daten, UBC-9-abhängige Signalwege als bedeutende Effektoren von EXO1 Stabilität identifizieren, was darauf hinweist, dass der Proteasom-vermittelte Abbau von EXO1 als Antwort auf stockende DNS-Replikation sumoylierungsabhängig ist.

Des Weiteren fanden wir, dass der UBC9-PIAS1/PIAS4 Signalweg EXO1 Proteinstabilität in vivo sowohl in basalem wie auch in DNS beschädigtem Zustand kontrolliert und wir waren in der Lage, EXO1 Sumoylierung in vitro mit purifiziertem, rekombinantem humanem oder Hefe-EXO1 als Substrate und Komponenten der Sumoylierungsmaschinerie wiederherzustellen.

Es zeigte sich, dass das de-sumoylierende Enzym SENP6 in vivo wie auch in vitro konstitutiv mit EXO1 interagiert und dass Abreicherung (depletion) von SENP6 den EXO1-Abbau fördert.

Wir demonstrierten auch, dass Sumoylierung und Ubiquitinierung sequenziell an EXO1 stattfinden, da durch störendes Eingreifen in das erstere durch UBC9 Abreicherung oder chemischer Inhibition des E1-SUMO aktivierenden Enzyms der zweite Mechanismus beeinträchtigt wird.

Später waren wir in der Lage zu zeigen, dass Sumoylierung nötig ist für die Rektrutierung von EXO1 an die DNS in Antwort auf Beschädigung, da UBC9 Abreicherung das Verhältnis von Chromatin-gebundenem zu freier EXO1 und die Lokalisierung von EXO1 an beschädigten Stellen senkt, was schliesslich in einer Senkung von EXO1-vermittelter Resektion von DNS-Enden resultiert.

In vitro Studien in Kombination mit massenspektrometrischen Analysen erlaubten die Identifikation der Lysin K_{655} und $K_{801/802}$ als wichtige

Sumoylierungsstellen in EXO1; Chromosome Spread Analysen von Zellen, die hohe Level Wildtyp EXO1 exprimieren, zeigten eine hohe Rate von Chromosomenbrüchen nach Camptothecin-Behandlungen. Dies war nicht der Fall bei Zellen, die ein SUMO-defizientes, mutante EXO1 exprimierten, was auf den wichtigen Mechanismus hindeutet, mit Hilfe dessen Krebszellen mit hochregulierter EXO1 Genexpression überleben können.

PART I

1. INTRODUCTION

1.1 DNA damage: causing and curing cancer

Cells are constantly subjected to DNA damaging events caused by exogenous and endogenous factors creating up to 10^5 DNA lesions per day. Exogenous factors include physical genotoxic agents, such as ionizing radiation (IR) occurring during treatments exposing the body to X-rays or ultraviolet (UV) light from sunlight. Also chemical agents such as methyl methanesulfonate (MMS) which attaches alkyl groups to DNA bases, mitomycicn C (MMC), cisplatin and nitrogen mustard which introduce covalent links between bases of the same DNA strand (intrastrand crosslinks) or of opposite DNA strands (interstrand crosslinks or ICLs), etoposide and camptothecin (CPT) which inhibit topoisomerase II and topoisomerase I, respectively, inducing the formation of single-strand breaks (SSBs) or double-strand breaks (DSBs) by trapping topoisomerase-DNA covalent complexes , represent external sources of damage [1]. Endogenous factors include, for example, reactive oxygen species (ROS) generated by the cells during metabolic processes; these, results in DNA breaks and spontaneous DNA alterations, such as dNTP misincorporation during DNA replication, DNA deamination, depurination and alkylation (Figure 1) [2, 3].

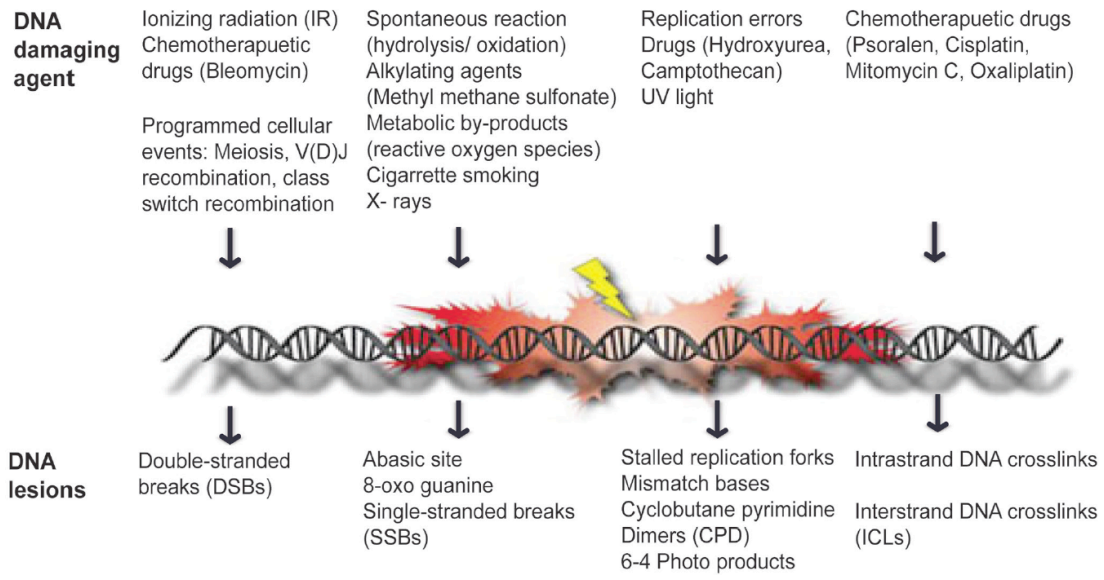

Figure 1. DNA damage: causes and effects. Endogenous and environmental sources of DNA damaging agent inflict damage to the DNA that range from modified bases, intra- and inter-strand crosslinks, cyclobutane pyrimidine dimers, 6-4 photoproducts, single- and double-stranded DNA breaks. Upon sensing DNA damage the cells activate the DDR network which activates cellular processes such as cell-cycle checkpoint control, transcription, DNA repair machinery, senescence and/or cell death. DNA repair pathways act independently or coordinate to repair DNA lesions. *Modified after (Ghosal, G. and J. Chen, DNA damage tolerance: a double-edged sword guarding the genome. Transl Cancer Res, 2013. 2(3): p. 107-129.)*

To counteract DNA damage and maintain genome integrity, cells need to adequately respond to such genotoxic stresses. This is achieved by activation of evolutionary conserved DNA-damage response (DDR) pathways that results in a two-pronged effect: a block in the cell cycle progression and the concomitant induction of DNA repair. Depending on the extent of damage suffered by DNA, cells may repair all lesions and re-enter the cell cycle, a condition known as checkpoint recovery or, in the case of excessive damage, be eliminated by programmed cell death (apoptosis). Alternatively, cells undergo an apparently permanent arrest after DNA damage. This state is known as "replicative senescence" and consists of an irreversible growth-arrest program that prevents unlimited cell proliferation. Although senescent cells in vitro may remain indefinitely viable, this may not be the case in vivo [4]. Genes coding for proteins that are involved in genome surveillance, such as DNA repair genes, are referred to as “caretakers”. Mutations or defects in such genes predispose an individual to cancer and may enhance tumorigenesis, a common condition in numerous hereditary cancer syndromes. Affected individuals inherit a defective allele of a caretaker gene and eventually acquire a mutation of the second allele in a somatic cell, an event that facilitates tumour survival and disease progression. Examples of inherited mutations in caretaker genes are those of the WRN DNA-helicase that are linked to the development of lymphomas or mutations in the BLM DNA-helicase that result in a leukemia-prone phenotype and mutations in the BRCA1 or BRCA2 genes that lead to increased incidences of breast and ovarian cancers, respectively [5].

Proper DNA replication is necessary to assure inheritance of the correct

genetic information to daughter cells and maintain genome stability. Genomic instability arising from the above-mentioned events is at the base of tumorigenesis but it is also used in cancer therapy to induce DNA lesions and apoptotic pathways in cancer cells. Indeed, there is evidence supporting the idea that DNA alterations during replication occur at early stages of tumorigenesis in many types of cancers promoting the activation of oncogenes, genes inducing over-proliferation [6]. In precancerous cells, such lesions are counteracted by a powerful DNA damage response (DDR) which induces senescence and apoptosis [7, 8] Upon further genetic and epigenetic rearrangements down-regulating the DDR, tumorigenesis progression takes place [9] as confirmed by the cancer-prone phenotype of many DDR syndromes (Table 1).

Table 1 Genetic disorders and cancer phenotype associated with defects in DDR pathway

DDR Defect	Mutated gene	Syndrome	Cancer predisposition
BER	MYH	MYH-associated polyposis	Colorectal cancer
MMR	MSH2, MSH6, MLH1, PMS2	Hereditary nonpolyposis colorectal cancer (HNPCC)	Colorectal cancer, carcinomas
NHEJ	RAG1, RAG2	Severe combined immunodeficiency (SCID)	
	XLF	Immunodeficiency with microcephaly	
	ARTEMIS	Radiosensitive severe combined immunodeficiency (RS-SCID)	Lymphomas
	LIG4	Ligase IV syndrome	Lymphomas
NHEJ, SSB repair	PNKP	Microcephaly intractable seizures and developmental delay syndrome (MCSZ)	
Class switch recombination (CSR)	AID, UNG	Hyper- IgM syndrome	
SSB Repair; NER	LIG1	Ligase I Syndrome	
SSB Repair	APTX	Ataxia with oculomotor apraxia 1 (AOA1)	
	SETX	Ataxia with oculomotor apraxia 2 (AOA2)	
	TDP1	Spinocerebellar ataxia with axonal neuropathy (SCAN1)	
TC-NER	CSB, XPD, XPG, ERCC1	Cerebo-oculofacio-skeletal syndrome (COFS)	
	CSA, CSB, XPB, XPD, XPG	Cockayne syndrome (CS)	
	XPB, XPD, TTDA	Trichothiodystrophy (TTD)	
NER	XPA, XPB, XPC, XPD, XPE, XPF, XPG, POLH	Xeroderma pigmentosum (XP)	Squamous and basal cell carcinoma, melanoma
NER, ICL repair	XPF	XPF-ERCC1 syndrome (XFE)	
ICL repair, HR	FANC-A, -B, -C, FANCD1 (BRCA2), FANCD2, -E, -F, -G, -I, FANCJ (BACH1), FANCL, FANCN(SLX4)	Fanconi Anemia (FA)	AML, squamous cell carcinoma, myelodysplasia
	RAD51C (FANCO)	Fanconi anemia-like disorder	
	FAN1	KIN (Karyomegalic interstitial nephritis)	
HR	ATM, BRCA1, BRCA2, BRIP1, CHK2, NBS1, PALB2, RAD50, RAD51C	Familial breast cancer	Breast and ovarian cancer
	BLM	Bloom Syndrome (BS)	Carcinoma, lymphoma, leukemia
	RECQL4	Rothmund Thomson syndrome (RTS)	Skin cancer, osteosarcoma
	WRN	Werner syndrome (WS)	Sarcoma
DNA replication	POLD1, POLE		Colorectal adenocarcinoma
Telomere maintenance	DKC, TERC	Dyskeratosis congenital (DKC)	Carcinoma
DNA damage signaling	Ribonuclease H2 (RNASEH2A, RNASEH2B, RNASEH2C), TREX1, SAMHD1	Aicardi Goutieres syndrome (AGS)	

Table 1 (*Continued*)

Table 1 (Continued)

DDR Defect	Mutated gene	Syndrome	Cancer predisposition
DNA damage signaling, DSB repair	ATM	Ataxia telangiectasia (A-T)	Leukemia, lymphomas, breast cancer
	MRE11	Ataxia telangiectasia-like disorder (A-TLD)	
	NBS1	Nijmegan breakage syndrome	B cell lymphoma
	RAD50	Nijmegan breakage syndrome-like disorder (NBSLD)	
	RNF168	Riddle Syndrome	
	TP53	Li-Fraumeni syndrome (LFS)	Brain and breast cancer, sarcomas
DNA damage signaling, DSB repair, replication fork repair	ATR, PCTN, SCKL2, SCKL3	Seckle syndrome (SS)	AML
	MCPH1	Primary microcephaly 1	
Replication fork repair	SMARCAL1	Schimke immunoosseous dysplasia (SIOD)	
Mitochondrial DNA maintenance	POLG, TWINKLE	Spino-cerebellar ataxia epilepsy syndrome (SCAE)	
	POLG, POLG2, TWINKLE, RRM2B	Progressive external ophtalmoplegia (PEO)	

Table 1. *(Modified after (Negrini, S., V.G. Gorgoulis, and T.D. Halazonetis, Genomic*

*instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol, 2010. **11**(3): p. 220-8.)*

In such cases, senescence and apoptosis bypass is facilitated despite accumulation of DNA damage [10]. In general we can say that cancer is fueled by genomic instability since most of cancers are characterized by chromosomal instability (CIN), accumulation of DNA base mutation and microsatellite instability (MIN) [9].

On the other hand, cancer cells are usually more susceptible to genotoxic agents compared to normal cells, likely due to the fact that first, they divide more rapidly and second, cancer cells usually carry alterations in some components of DNA repair pathways that arise during tumour development. Another important consideration in this respect is that the high proliferation rate of cancer cells and the associated metabolic stress, like hypoxia and mitotic stress, renders them more reliant on anti-stress mechanisms, such as DDR pathways [11]. Thus, combinatorial treatments with conventional DNA-damaging chemotherapeutic drugs and inhibitors of specific DNA repair pathways, might be the best choice in the treatment of cancer. Moreover, few years ago, it was observed that many non-oncogenic (NOA, non-oncogenic addiction) pathways are of vital importance for cancer cells, despite such pathways are not essential for normal cells survival. Thus, targeting NOAs represents a further step towards the selective growth repression of cancer cells [10].

To conclude, exposing to light the events that cause DNA lesions and the peculiar and precise mechanisms evolved by cells to repair them, will facilitate the understanding of tumorigenesis.

1.1.1 Replication stress

Replication fork stalling represents the initial event which, if not properly solved, may lead to failure in proper duplication of the genetic information and thus to chromosomal aberrations or mitotic catastrophe, resulting in tumorigenesis or apoptosis, respectively. Particular regions of the genome, known as common fragile sites (CFSs), are more prone to breakage than others upon exposure of cells to replicative stress; such regions are the first

being damaged [6]. Other regions of the genome, called early-replicating fragile sites (ERFSs) seem to be replication stress sensitive; these segments are located in highly transcribed, repetitive and CpG-rich regions where replication-transcription collisions create lesions [12]. When DNA replication slows down because of the presence of the lesion, multiple mechanisms act to sense the damage, stabilize and restart the stalled forks [13].

1.1.2 Double-strand breaks (DSBs)

Double-strand breaks represent the most toxic DNA lesions since they can trigger profound genomic rearrangements or generate genetic modifications of essential biological processes. Harmful cases are those in which the DSB is the result of exposure of DNA to exogenous or endogenous stresses leading to replication forks stalling and collapse. In other cases, DSBs are physiologically induced by cellular enzymes in processes like meiotic differentiation, mating-type switching in *Saccharomyces cerevisiae* [14] or in V(D)J and class switch recombination to promote the diversity of the immune response system [15].

1.1.2.1 Hydroxyurea-induced DNA damage

Hydroxyurea (HU) is a powerful inhibitor of the ribonucleotide reductase (RNR) enzyme that catalyzes the formation of deoxyribonucleotides, used in the synthesis of DNA, from ribonucleotides [16]. HU treatment of cells leads to starvation of DNA polymerase at replication forks for dNTPs, reducing, and only rarely completely depleting, the purine bases dGTP and dATP pool [17]. Changes in individual dNTP pools were observed in HU-treated mammalian cells, likely due to the compensatory activity of deoxyribonucleotide saving pathway typical of higher eukaryotes. Budding yeast represent an easier system to study HU-dependent effects on replication since they do not possess any deoxyribonucleoside kinase activities and they completely rely on ribonucleotide reductase to synthesize deoxyribonucleotides [18]. Previous studies suggested that HU-treatment does not exhaust the dNTP pools most

likely because cells evolved mechanisms in order to arrest DNA replication as soon as the pool of deoxyribonucleotides reaches a minimum threshold, likely to conserve basal dNTPs for processes such as DNA repair. Indeed, it was shown that nucleotide excision repair pathway is sensitive to dNTP pool depletion in cultured human fibroblasts [19]and moreover, HU-treated yeast cells complemented with artificial dNTP pools showed increased cell survival upon DNA damage [20].

1.1.2.2 Camptothecin-induced DNA damage

Camptothecin (CPT) is a cytotoxic antitumor drug; it interferes with DNA replication by inhibiting the activity of Topoisomerase I, an enzyme required to remove the super helical tension accumulating in front of advancing replication forks [21]. CPT stabilizes the so-called 'cleavable complexes' between Topoisomerase 1 and the 3' broken DNA end [22]. Indeed, Topoisomerase I releases topological stress by breaking the DNA double helix on one strand and passing the other strand through the break until re-ligation of the 5' rotating end to the 3' end [23]. CPT-treated mammalian cells were shown to generate replication forks-associated DNA double-strand breaks resulting in extended and perhaps permanent cell cycle arrest in S/G2 phase upon removal of the drug, likely due to the presence of unrepaired DNA breaks [24]. CPT activity is specifically toxic during S-phase, although the levels of Topoisomerase 1 and cleavable complexes are relatively constant throughout the cell cycle. It was suggested that there is some interaction between the cleavable complexes and the moving replication machinery in S-phase resulting in irreversible fork arrest and the conversion of the cleavable complexes to an irreversible enzyme-linked DNA strand-break. These breaks will collapse into double-strand breaks, highly toxic lesions.

1.1.3 DSBs signaling

When a DSB occurs on DNA, a number of different proteins are recruited to the site of damage in a time- and space-highly coordinated way. MRE11,

RAD50 and NBS1 proteins, members of the MRN complex in mammalian cells, are among those proteins first sensing the lesion and being recruited on chromatin. MRN is a highly conserved protein complex and it is essential for cell survival, since null mutations in any of the components result in embryonic lethality in mice [25]. Indeed, MRN-defective syndromes are characterized by mutation, for example, in MRE11 gene (ataxia-telangiectasia-like disorder or ATLD) [26], in NBS1 gene (Nijmegen breakage syndrome) or in *RAD50*, leading to a variant form of Nijmegen breakage syndrome (NBS-variant) [27]. The MRN complex first binds and processes broken DNA ends leading to recruitment and activation of the ATM (ataxia-telangiectasia mutated) kinase [28]. In response to DSBs, ATR (ataxia-telangiectasia and Rad3-related) kinase activity is triggered in an ATM-dependent manner in the S and G2 phase of the cell cycle [29]. Activated ATM phosphorylates H2AX at the conserved C-terminal S139 residue in response to DSBs [30]. H2AX phosphorylation occurs on megabase regions flanking the DSBs within seconds after the DNA damage occurs, suggesting that H2AX phosphorylation may be a critical event in early DNA damage signaling [31]. One key function of phosphorylated H2AX (γ-H2AX) is to provide a high-affinity binding site for MDC1 (mediator of DNA damage checkpoint 1), which binds γ-H2AX via its C-terminal tandem BRCT repeats. MDC1 functions as a molecular bridge between γ-H2AX and the NBS1 component of the MRN complex, and helps providing a platform for various dynamic interactions for these and additional checkpoint and DNA repair proteins within the vicinity of the damage sites. Moreover, this interaction protects γ-H2AX from de-phosphorylation [32]. The key function of activating ATM/ATR signaling is the initiation of cell cycle arrest at G1/S, intra-S-phase and G2/M checkpoints. ATM phosphorylates the checkpoint kinase-2 (CHK2) at Thr-68, while ATR phosphorylates the checkpoint kinase-1 (CHK1) at Ser-317 and Ser-345 [33]. Specifically, ATM/ATR-dependent phosphorylation of CHK2 and CHK1 facilitates intra-molecular auto-phosphorylation leading to full checkpoint kinase activation and further transmission of the signal to key cell-cycle regulators, including the CDC25 family of phosphatases, the kinase WEE1 and p53, which itself can undergo phosphorylation by ATM at Ser-15. Temporal cell-cycle arrest induced in response to DNA damage is established

through activation of the kinase WEE1 and inhibition of CDC25 activity, which results in inactivation of cyclin-dependent kinases (CDKs) at different stages of the cell cycle. A more sustained arrest is instead provided by p53-induced transcription of the CDK inhibitor p21 [34]. Cell cycle arrest presumably provides time to allow DNA repair to occur before the lesions are encountered by a replicative polymerase during DNA replication as well as to prevent the mis-segregation of chromosomal fragments during anaphase.

1.1.4 DSBs repair

Classically, two conceptually different mechanisms can in principle repair DSBs occurring in the genome of higher eukaryotes: Non-homologous end joining (NHEJ) and homologous recombination repair (HR) [35]. As their names imply, NHEJ simply restores integrity in the DNA by joining the two ends without necessarily preserving the original sequence. As a result, it is error-prone. Because a second DNA molecule is not required for the function of this repair pathway, it is active throughout the cell cycle. HR, on the other hand is equipped to maintain fidelity in the sequence of the DNA molecule. To achieve this, HR requires an undamaged homologous sequence that serves as template for repair of the broken strands. There are two sources of homology in mammalian cells. The homologous chromosome that is present throughout the life cycle of the cell and the sister chromatid that is generated after DNA replication and which, therefore, exists only during the S and G2 phases of the cell cycle. Accumulating evidence supports the view that HR uses the sister chromatid as template rather than homologous chromosomes, a requirement that restricts the function of this pathway to the S and G2 phases of the cell cycle. This requirement probably derives from the fact that in a eukaryotic cell nucleus the homologous chromosomes are accommodated in distinct and frequently distantly located domains that renders difficult the search for homology (a key step of HR). In addition, using sequences on the homologous chromosome as template carries the implicit risk of generating gross chromosomal rearrangements (GCRs) such as deletions, inversions or loss of heterozygosis [36].

The fact that at least two genetically and conceptually distinct repair pathways are involved in the repair of DSBs raises questions regarding their coordination. If these pathways operate independently of each other it is possible that they compete against each other. On the other hand, if they collaborate, then the question is how their functions are coordinated. As stated above, the choice between either pathway depends on the phase of the cell cycle. Studies of either NHEJ- or HR-deficient cells suggest that these two pathways compete for the repair of DSBs [37]. HR-deficient cells have a significant DSB repair defect only during the S/G2/M phases, whereas NHEJ-deficient cells showed reduced repair efficiency at all cell cycle stages [38]. The mechanisms by which cells decide between these two repair pathways have been studied for the past few years. According to the evidence obtained so far, DNA-end resection is a critical step that favors the choice of HR over NHEJ and it is regulated by cyclin-dependent kinases (CDKs), the master regulators of the cell cycle [30]. Analysis of end resection in yeast revealed that inhibition of CDK1/cdc28 in the G2-phase prevented end resection and checkpoint activation as well persistence of Mre11 at the DSB site, consistent with the idea that processing of the break had stalled. This suggests that CDK1 controls Mre11-associated nuclease function at the DSB, but does not influence its recruitment to DNA ends [39]. In addition, Sae2, a DNA endonuclease that controls the initiation of DNA end resection in yeast [34] is regulated by CDK-dependent phosphorylation. Mutation of Sae2 Ser-267 to the non-phosphorylatable residue alanine (S267A) caused an end-processing phenotype comparable with deletion of Sae2. In contrast, mutating the same residue into a residue that mimics constitutive phosphorylation (S267E) complemented these phenotypes and bypassed the need for CDK activity in DSB end resection [40]. The Sae2-null and S267A mutants showed delayed HR and enhanced NHEJ, whereas the S267E mutant showed slightly enhanced recombination and a decrease in NHEJ. This indicates that CDK1/cdc28-mediated phosphorylation of Sae2 in yeast regulates the balance between HR and NHEJ during the cell cycle. The motif of Sae2 that contains the residue Ser-267 is highly conserved amongst orthologs in higher eukaryotes, and mutation of the analogous residue in human CtIP also resulted in hypersensitivity to camptothecin [40]. These results suggest that

similar CDK control of DNA end resection exists in other organisms. Moreover, it was shown that phosphorylation of a putative CDK site in human CtIP [CTBP (C-terminus-binding protein of adenovirus E1A)-interacting protein] enables it to interact with the BRCA1 C-terminal tandem BRCT domain, an interaction that is required for efficient end resection [41]. This, in turn, suggests that the BRCA1-CtIP interaction influences the balance between HR and NHEJ. Collectively, these results support a model in which the commitment to DSB end resection and repair is regulated in order to ensure that the cell activates the most appropriate DSB repair pathway to optimize genome stability (Figure 2).

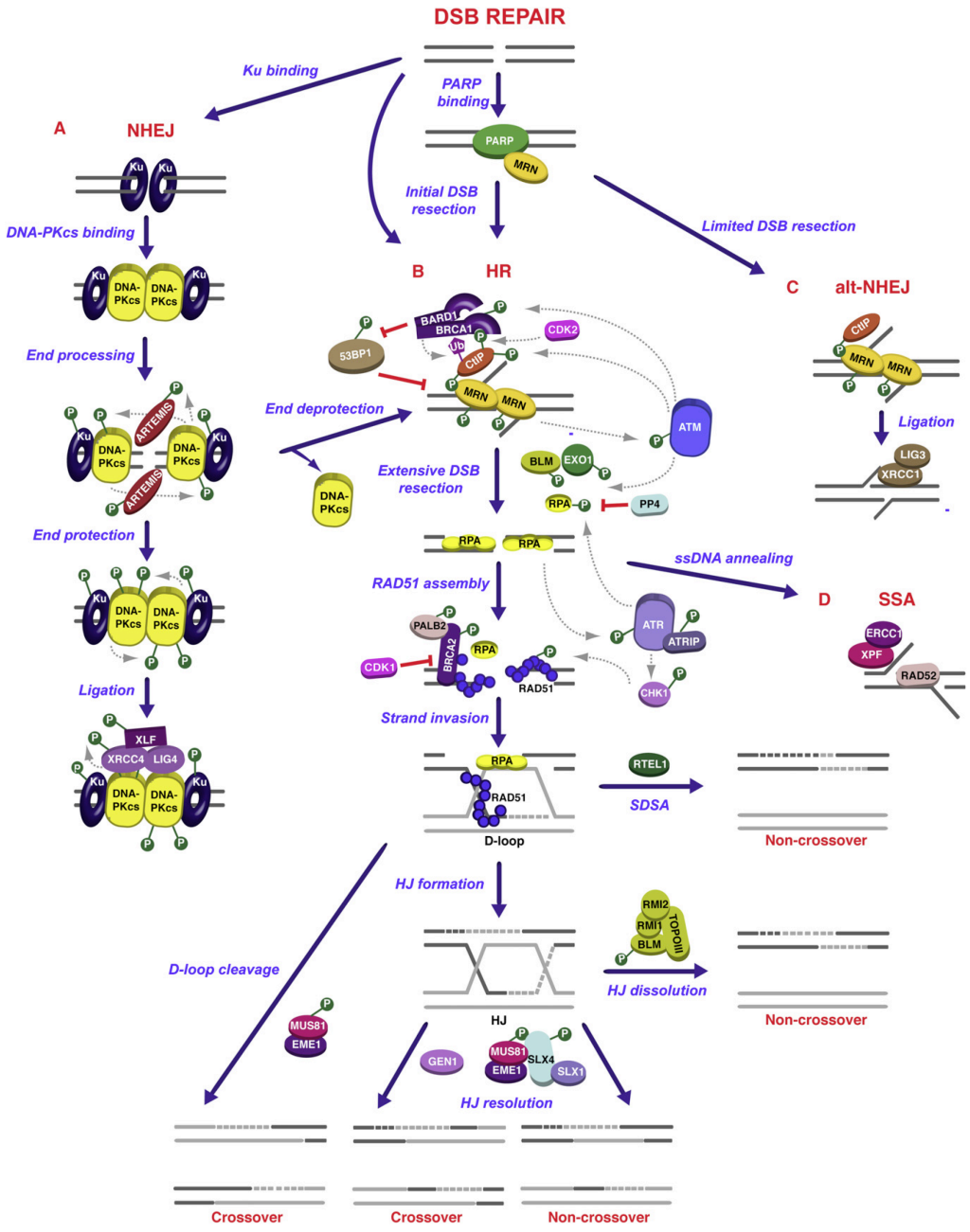

Figure 2. DNA repair pathways involved in the repair of double-strand break. (A) Rapid association of Ku to DSBs promotes NHEJ by recruiting DNA-PKcs. Sequential phosphorylation events on multiple DNA-PKcs amino acid clusters favors the initial processing of DNA ends by ARTEMIS, followed by DNA-PKcs-dependent protection of DNA ends required for DNA ligation. (B) Alternatively to NHEJ, MRN, which is initially recruited to DSBs by PARP in competition with Ku, mediates the initial stages of DSB resection together with CtIP and BRCA1 to promote homologous recombination in S and g2. 53BP1 has an inhibitory role on DSB resection and is negatively regulated by BRCA1 by unknown mechanisms. The MRN/CtIP/BRCA1 complex can also promote DSB resection following de-

protection of DNA ends when NHEJ fails. Extensive DSB resection and formation of RPA-coated 3' ssDNA ends is induced by EXO1 and BLM. ATM plays a central role in the regulation of DSB resection. Displacement of RPA from the 3' ssDNA ends and assembly of RAD51 filaments mediated by BRCA2 leads to strands invasion into homologous DNA sequences. Recruitment of RAD51 to ssDNA ends is regulated by the ATR pathway, which is activated following DSB resection. D loop structures formed after strand invasion can be cleaved by MUS81/EME1 or displaced by RTEL1 during SDSA to generate crossover events, respectively. Noncrossovers are generated also by dissolution of Holliday junctions (HJs) by the BLM/TOPOIII complex, whereas HJ resolution by the nucleases GEN1 and SLX1/SLX4, which associates with MUS81/EME1, can generate both crossover and noncrossover events. (C) Limited DSB resection carried out by CtIP and MRN in G1 results in alternative NHEJ. (D) Following DSB resection, 3' ssDNA ends with homologous sequences can be directly annealed by RAD52. *Modified after (Ciccia, A. and S.J. Elledge, The DNA damage response: making it safe to play with knives. Mol Cell, 2010.* ***40****(2): p. 179-204.)*

1.1.4.1 Homologous recombination (HR)

The goal of HR is to facilitate recovery of information lost as result of damage to both DNA strands by retrieving it from an undamaged homologous sequence. To this end, damaged and undamaged DNA molecules need to directly interact. In particular, the damaged DNA molecule will need to first undergo processing in order to generate DNA structures that can “read-off” sequence information. Furthermore, the chromatin structure on both DNA molecules will need to be modified in order to facilitate the search for the homologous sequences in the sister DNA molecule. Once homology has been found, sequence information will need to be copied and finally the interacting DNA molecules will need to be separated.

HR starts with the resection of DNA ends around the DSB, forming 3’-single stranded DNA (ssDNA) stretches [42]. This form of DNA can invade and pair to homologous sequences present in an intact DNA molecule and is also suitable to be extended by DNA polymerases to copy the missing sequence information. In cells of higher eukaryotic organisms the initial resection of DNA ends is orchestrated by the MRN complex [43], assisted by the function of the resection-promoting factor CtIP [44-46]. These proteins collaborate to trim the DNA ends to an intermediate form in a process so-called “short-range” resection. The trimmed DSB is then resected more extensively in a step of

“long-range” resection [30, 44]. Possible candidates are the exonuclease-1 (EXO1) and the Bloom’s syndrome protein (BLM) together with the nuclease DNA2 [46]. The generated single-stranded 3’-overhangs are coated by the replication protein A (RPA) heterotrimer, the major mammalian ssDNA binding protein. This rapid binding by RPA is believed to protect the ssDNA and to prevent the formation of secondary DNA structures [47]. In addition, RPA also mediates the recruitment of the ATR/ATRIP complex to the single stranded regions and initiates the DDR signaling cascades, which among others inhibit cell cycle progression through activation of the corresponding checkpoints [48].

The subsequent DNA strand invasion and homology search requires the formation of a nucleoprotein filament composed of Rad51 bound to ssDNA. Since RPA binds more avidly to ssDNA than Rad51, additional activities are required to load Rad51 onto RPA-coated ssDNA and to displace RPA. In mammalian cells, an important mediator complex is BRCA1/BARD1 and BRCA2 (FANCD1)/DSS1, bridged by the PALB2 (partner and localizer of BRCA2) (FANCN) [49]. The direct loading of Rad51 is believed to be through its direct interaction with BRCA2 [50]. This interaction is thought to be limited to S and G2 phases of the cell cycle by CDK-dependent phosphorylation of BRCA2 [51]. The Rad51 nucleoprotein filament then invades a duplex DNA of the sister chromatid and searches for homology. Once found, the invading strand sets up a structure, which involves pairing with the complementary strand and displacement of the other, resulting in a so-called D-loop (displacement loop) structure. At this point, HR can be completed via several pathways and different outcomes. Synthesis-dependent strand annealing (SDSA) or double strand break repair (DSBR) have been described to occur in case of two-ended DSBs. In SDSA the elongated invading strand pairs with the second DSB end. This process only produce non-crossovers, hence SDSA is the preferred recombination-mediated pathway for DSB repair in somatic cells to prevent loss of heterozygosity. Alternatively, during DSBR, the D-loop gets extended and captures the second DSB end, creating a double Holliday junction (HJ) between the four strands that can undergo branch migration, a process catalyzed by members of the RecQ helicase family [52]. HJ intermediates can be resolved in different ways resulting in

either non-crossovers or crossovers, the later being predominant during meiotic recombination. The BLM/Topo IIIα can dissolve HJ to form non-crossover products [53]. Alternatively, the MUS81-EME1 complex may cleave HJs to yeild crossovers [54]. Two more HJ resolvases that has been recently identified in humans cells, GEN1 and SLX1/SLX4 promotes the resoultion of HJs by a mechanism that is believed to generate crossovers and non-crossovers [55-57].

In the abscence of a homology, single-strand annealing (SSA) can be the pathway of choice. SSA involves the exposing of repetitive sequences by resection of both 5'-strands until sequences are uncovered and annealed. DNA flaps are then removed by nucleases followed by DNA synthesis and ligation of the nicks. Since SSA involves the deletion of the intervening sequences, it is considered as a mutagenic repair pathway. One-ended DSBs generated through uncapping telomeres or collapsed replication forks after encountring a single strand break (SSB) or nick, are repaired by break-induced repair (BIR). In BIR, the invading 3'-strand forms a replication fork that copy long tracts from the donor DNA molecule. This process could potentially lead to loss of heterozygosity.

1.1.4.2 Non-homologous End joining (NHEJ)

NHEJ is the major pathway for DSBs repair in mammalian cells. It involves joining the two ends of a DSB through a process largely independent of homology. NHEJ provides a relatively simple mechanism for the repair of DSBs throughout the cell cycle, but of particular importance during G0-, G1-, and early S-phase [58]. NHEJ only works efficiently and with high fidelity in the repair of DSBs displaying complimentary overhangs, 5' phosphates and 3' hydroxyl groups, so called 'clean' DSBs, such as those produced by nucleases. In yeast and mammalian cells, approximately 25-50% of nuclease DSBs is repaired by precise NHEJ [59]. If the ends are not compatible, then processing is required and this can result in mutagenic deletions or insertions at the break site [60]. The molecular mechanism by which NHEJ operates could be simplified in three main steps: first both ends of the broken DNA are

captured, second a molecular bridge is formed which brings the two DNA ends back together, finally the broken DNA ends are re-ligated.

Central to NHEJ in organisms from yeast to man is the Ku protein, a heterodimer of two subunits called Ku70 and Ku80 [61]. Biochemical studies of mammalian Ku have showed that it can bind DNA in a non-sequence-dependent manner and that binding is dependent on DNA DSBs [62]. The NHEJ process is initiated by the binding of the Ku70/80 heterodimer to both ends of the broken DNA molecule, forming a ring-shaped structure, in which the opening of the ring accommodates a DNA helix. This feature allows the Ku heterodimer to slide over the ends of a broken DNA molecule [63]. It is believed that the association of a DNA end with the Ku heterodimer creates a platform for the assembly of other NHEJ key enzymes and proteins. In vertebrates, Ku serves as the DNA targeting subunit for the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which together with the Ku forms the DNA-PK holoenzyme. The association of the DNA-PKcs with DNA activates its serine/threonine kinase activity. Among the different phosphorylation targets of DNA-PKcs are XRCC4, the nuclease Artemis and DNA-PKcs itself, which can undergo autophosphorylation. This autophosphorylation is believed to influence its conformation and dynamics, serving to relieve blockage of the ends by DNA-PKcs, hence facilitating access of other repair factors [64]. Occasionally, the generated DSBs might require further processing to generate ligatable 5'-phosphorylated ends in order for repair to be completed. The nuclease Artemis, a single-stranded 5'-3' exonuclease, which upon phosphorylation by DNA-PKcs acquires an additional endonuclease activity specific for hairpins and ssDNA overhangs, as well as the polynucleotide kinase (PNK), together with DNA-PKcs have been shown to stimulate DNA-processing for efficient NHEJ [65]. The processed DNA-ends might lead to the generations of DNA gaps that are filled in by DNA polymerases. Members of the DNA polymerase X family of polymerases, including polymerase μ, polymerase λ and terminal deoxyribonucleotidyltransferase (TdT), have been shown to fill the gaps generated during NHEJ. Finally, NHEJ is completed by ligation of the DNA ends, a step carried out by the NHEJ ligase complex (also known as X4-L4), which is composed by XRCC4, DNA ligase IV and XLF [59].

Alternative NHEJ acts in the absence of classical NHEJ factors such as Ku, XRCC4 or DNA ligase IV. There, repair events involve small deletions and require short stretches of homology between the ligatable DNA ends. Microhomology-mediated end-joining (MMEJ) is the dominant pathway during alternative end-joining. In this repair mechanism DNA is slightly resected (less than 100 nucleotides) to expose regions of homology, which lead to reattachment of the two DNA ends of the break. The DNA is further processed by nucleases that remove flaps and overhangs and eventually by DNA polymerases that fill in the gaps [66].

1.2 The DNA damage response (DDR)

DNA lesions and replication stress are sensed by the DNA Damage Response (DDR) signal transduction cascade, which leads to the repair of damage and allows cells to proceed through the cell cycle [61]. In eukaryotic cells, the DDR uses signal sensors, transducers and effectors (Figure 3).

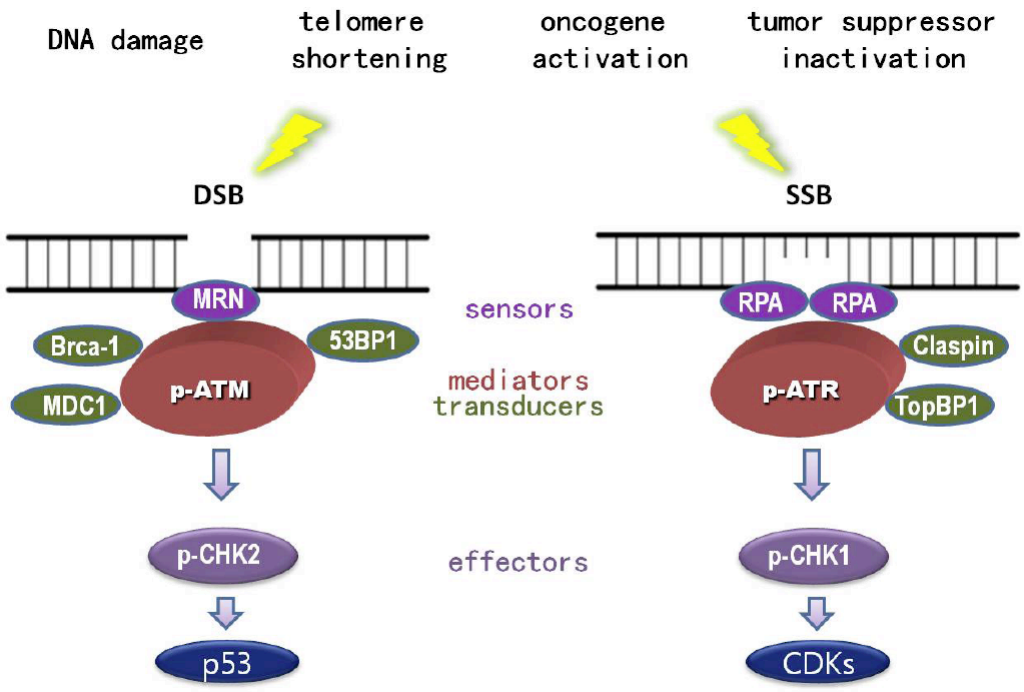

Figure 3. DNA damage and DNA damage response (DDR). Both external insults and internal hazards can cause DNA damage. DNA damage response is coordinated by various proteins whose function can be categorized as DNA damage sensors, transducers, mediators and effectors. Double strand DNA damage (DSB) can be detected by MRN complex (sensor) to recruit and activate transducer ATM (Ataxia Telangectasia Mutated) to activate CHK2 (effector), with the help of DDR mediators MDC1 (mediator of DNA damage checkpoint), 53BP1 (p53-binding protein 1), and BRCA1 (breast cancer 1). In contrast, single strand DNA damage (SSB) could be detected by sensor protein (replicative protein A), to recruit and activate transducer ATR (Ataxia Telangectasia- and Rad3-related), to activate CHK1 (effector), with the help of mediators TopBP1 (topoisomerase-binding proteins 1) and Claspin. p53 and CDKs are the major downstream substrates in response to DSB and SSB respectively. Modified after (Li, X., et al., The yin-yang of DNA damage response: roles in tumorigenesis and cellular senescence. Int J Mol Sci, 2013. **14**(2): p. 2431-48.)

The sensors include those proteins directly recognizing the aberration on DNA and activating the most upstream DDR kinases ATM and ATR. Such kinases represent the transducers promoting the series of phosphorylation events within the DDR network. The substrates of ATM and ATR represent the effectors, proteins involved in essential processes for genomic stability maintenance such as DNA replication, DNA repair and cell cycle control. In general we can define the DDR response mechanism as a phosphorylation-based transduction pathway, since the most upstream transducers are large serine/threonine kinases phosphorylating hundreds of proteins at Ser/Thr-Glu motifs [63].

DNA-damaging agents mediate the activation of the two main players starting the DDR cascade: ATM and DNA-PK. Together with ATR, they represent members of the phosphatidylinositol 3-kinase-like protein kinase (PIKKs) family and they act in conjunction with members of the poly(ADP-ribose) polymerase (PARP) family [61]. Unlike ATM, which regulates many different substrates, DNA-PK acts on a small group of proteins involved in DSB ends re-joining. ATR is activated only in a second step of the repair process, in complex with its partner protein ATRIP [52]. The PARP family has 16 members but only two of them are involved in the DDR, PARP1 and PARP2; they are activated upon SSBs and DSBs and their activity consists in the attachment of poly (ADP-ribose) chains on proteins as a signal for other DDR factors to be recruited on chromatin at the sites of damage (Figure 4).

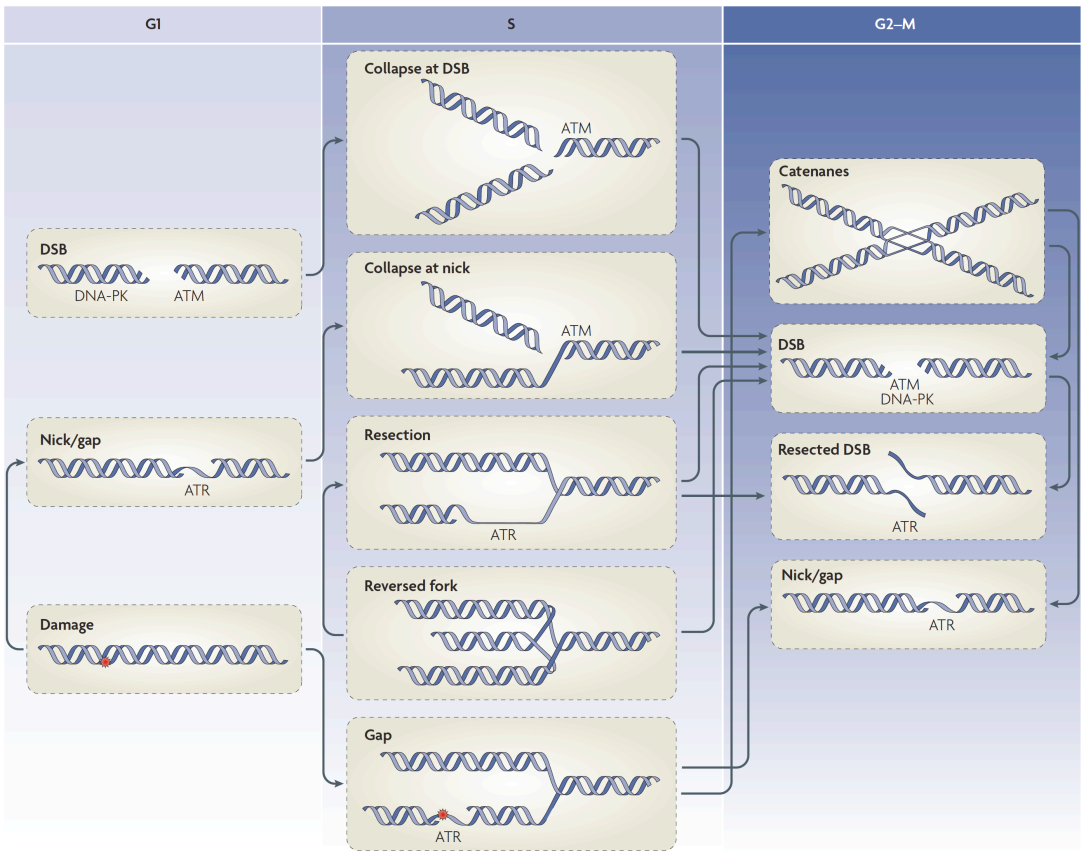

Figure 4. Cell-cycle-specific DNA structures and lesions and the checkpoint kinases that respond to them. During G1 phase, double-strand creaks (DSBs) lead to activation of the phosphoinositide 3-kinase related kinases DNA-PK and ATM, whereas other types of damage, such as ultraviolet-induced pyrimidine dimers, are processes by nucleotide-excision repair enzymes and lead to ATR activation. DSBs or nicks that are not repaired during G1 result in collapse of replication forks, which activates ATM (following DSBs resection, ATR is also activated). S-phase DNA damage, such as stalled forks or gaps that are generated during replication, activate ATR. In pathological conditions, for example, when cells contains mutations in genes of the ATM-ATR pathway, accumulation of reversed forks is processed by nucleases that lead to extensive gaps or DSBs. Topological problems during replication can form catenanes, which can result in nicks or, if unresolved, can lead to DSBs during chromosome segregation. *Modified after (Branzei, D. and M. Foiani, Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol, 2008.* ***9****(4): p. 297-308.)*

1.2.1 ATM and ATR Kinases specificity

The study of the two apical kinases ATM and ATR represent most of the knowledge collected until now about the DDR. Upon identification of the DNA lesion by sensor proteins, ATM and ATR phosphorylate DDR amplifying proteins which recruit the substrates of the two kinases [69]; such substrates can be phosphorylated directly by ATM/ATR or indirectly by CHK1 and CHK2 kinases which are themselves ATM/ATR targets [61]. The Triple T complex (TEL2-TTI 1-TTI 2) is responsible for ATM and ATR stability since it associates with the heat shock protein HSP90 and promotes the synthesis of new PIKKs. In-vivo and in-vitro studies suggested that different types of DNA lesions are sensed by ATM and ATR; ATM is primarily activated by double-strand DNA breaks (DSBs) while ATR activation arises upon a broader range of lesions interfering with DNA replication, also including DSBs.

In response to DSBs, ATM is activated by being recruited to the damaged sites where it phosphorylates its direct substrates BRCA1, CHK2 and p53 and mediates the consequent DNA repair cascade activation, cell-cycle arrest, apoptosis and other downstream pathways [67]. As previously reported, ATM is a mediator in the DDR process and therefore it needs to be regulated by upstream sensor proteins, which, in the case of DSBs, are represented by Mre11-Rad50-Nbs1, members of the MRN complex.

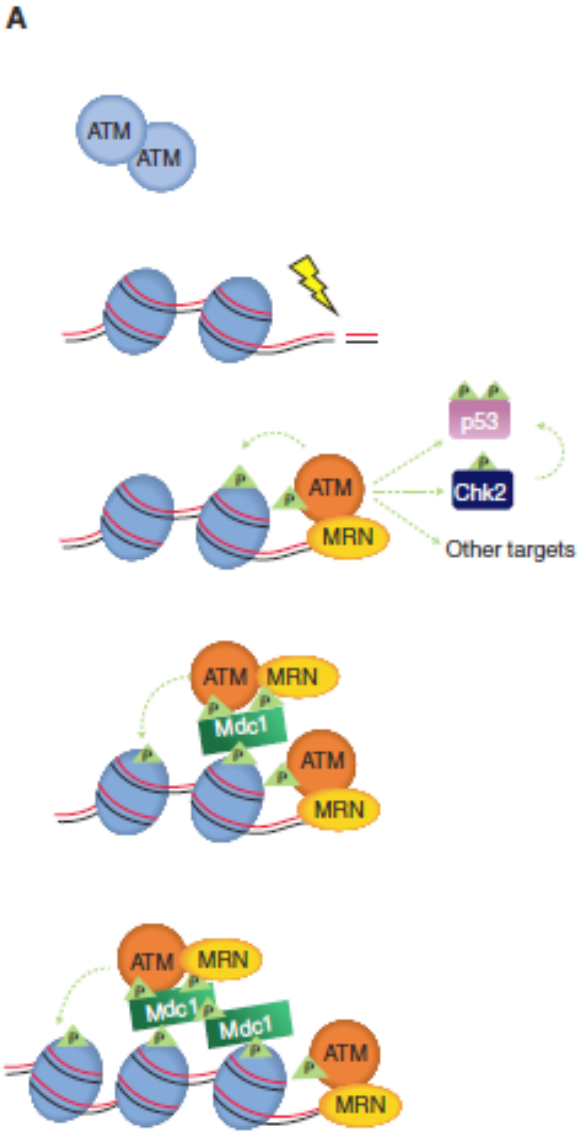

Figure 5. Activation of ATM by DSBs: Recognition of DNA ends and chromatin by ATM. The MRN complex functions as a sensor of DNA ends and activates ATM. The ATM activated by DNA ends (red) phosphorylates substrates such as CHK2 and p53, and the H2AX in flanking nucleosomes. Phosphorylated H2AX (γ-H2AX) is recognized by MDC1, which triggers a feed-forward loop that spreads activated ATM and γ-H2AX over large chromatin domains. *Modified after (Marechal, A. and L. Zou, DNA damage sensing by the ATM and ATR kinases. Cold Spring Harb Perspect Biol, 2013.* ***5****(9).)*

The exact mechanism by which MRN activates ATM is not yet completely understood, although in-vitro studies suggested that the presence of purified MRN proteins stimulates ATM kinase activity only in the presence of free dsDNA ends, blunt ends or also dsDNA ends with short single-strand DNA (ssDNA) overhangs, as binding substrate [28]. Specifically, it was also shown that the carboxyl terminus of Nbs1 directly interacts with ATM [68]. Following this initial activation step, ATM mediates the phosphorylation of the histone variant H2AX (γ-H2AX refers to the phospho-form of H2AX) which occurs within minutes after the DNA damage, spreading over >500 Kb chromatin domains flanking the lesion. This event is important for the recruitment of many DNA-repair proteins and chromatin-remodeling factors around the damaged site but not for phosphorylation of other ATM substrates [69]. In order to be able to phosphorylate H2AX contained in the nucleosomes flanking the lesion and

thus to be able to propagate the phosphorylation to other histones along the chromatin, ATM interacts with the FHA domain on MDC1, another mediator protein, which at the same time binds the previously phosphorylated γ-H2AX, so that ATM can keep phosphorylating the immediately adjacent histone. Auto-phosphorylation of ATM has been reported to be essential for the binding with MDC1 (Figure 5). ATR, the second kinase involved in the DDR pathway, is activated upon direct interaction between ATRIP, its partner protein, and RPA-coated ssDNA, a key structure generated upon DNA ends resection in response to DSBs occurring during S-phase of cell cycle [70]. Thus, we can say that ATR activation is resection-dependent and that lengthening of RPA-coated ssDNA switches the signal cascade from an ATM- to an ATR-activating mode. ATR-ATRIP recruitment to DNA promotes its own trans-autophosphorylation at T1989 residue and the additional recruitment of the RAD17-RFC2-5 clamp loads to junctions between ssDNA and dsDNA. The 911-checkpoint clamps, including RAD9-RAD1-HUS1 proteins, is then loaded on dsDNA. TOPBP1 interacts with RAD9 and Rhino, a protein interacting with the 9-1-1 complex, and it stimulates the ATR-ATRIP complex kinase activity (Figure 6). ATR substrates, CHK1 effector kinase, RPA and other targets are then phosphorylated. Moreover, CHK1 was shown to be phosphorylated consecutively in respect to CHK2 (ATM substrate), supporting the fact that ATR is involved in a second wave of activation [71, 72]. Activated CHK1, in turn, is essential for the intra-S and G2/M checkpoint responses and for RAD51 recombinase phosphorylation, important for the repair of DSBs in S-phase.

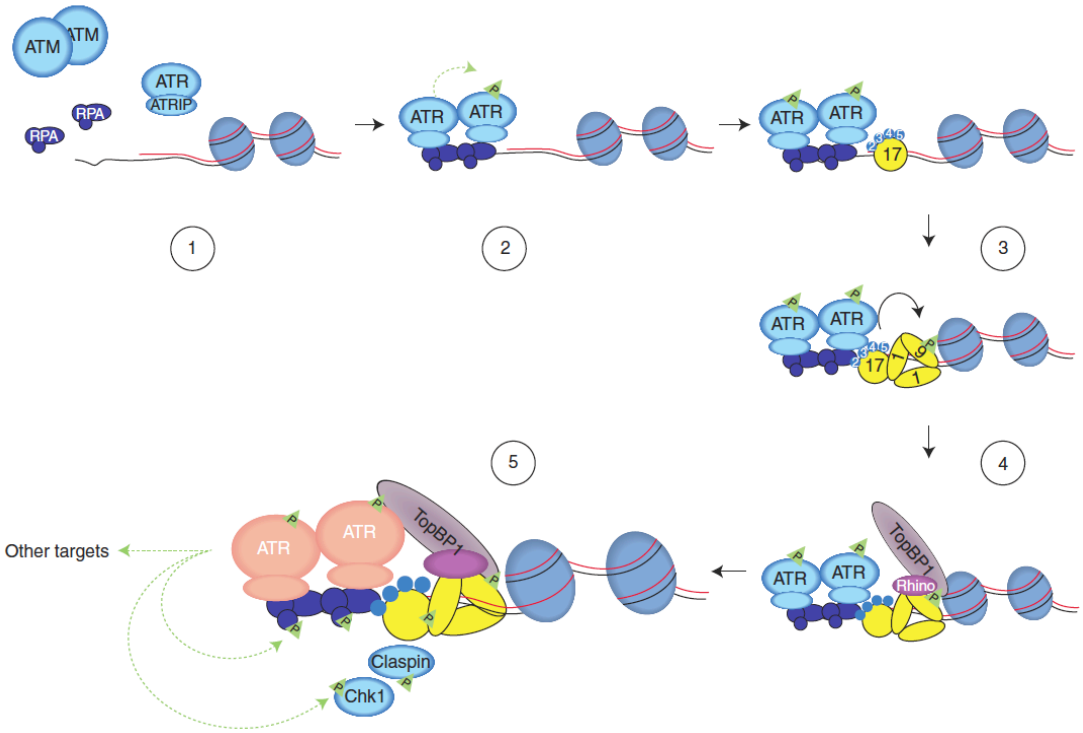

Figure 6. A fail-safe, multistep mechanism for ATR activation. Increased amounts of ssDNA are generated by resection of DNA ends or by uncoordinated DNA unwinding and synthesis at replication forks. Extensively resected DNA ends are no longer recognized by ATM efficiently. Once coated by RPA, ssDNA recruits the ATR-ATRIP complex (1), and promotes ATR trans-autophosphorylation (2). RPA-ssDNA also promotes the recruitment of the Rad17-Rfc2-5 clamp loader to junctions between ssDNA and dsDNA, and the loading of Rad9-Rad1-Hus1 (9-1-1) checkpoint clamps onto dsDNA (3). TopBP1 interacts with the phosphorylated Rad9 and with Rhino, which associates with 9-1-1 (4). the TopBP1 recruited to dsDNA by 9-1-1 and rhino engages the ATR-ATRIP complex on RPA-ssDNA through the ATR autophosphorylation site T1989. This process enables TopBP1 to stimulate ATR-ATRIP to its full capacity (pink) on ssDNA (5). TopBP1 may also function as a scaffold to facilitate ATR substrate recognition. This multistep process for ATR activation ensures that ATR is only activated when both ssDNA and ssDNA/dsDNA junctions are present at sites of DNA damage and are recognized by DNA damage sensors, providing a fail-safe but reversible mechanism to signal DNA damage. The dashed green lines represent phosphorylation events, and the solid black line represents the loading of 9-1-1 by Rad17-RFC2-5 complex. *Modified after (Marechal, A. and L. Zou, DNA damage sensing by the ATM and ATR kinases. Cold Spring Harb Perspect Biol, 2013.* ***5****(9).)*

1.2.2 Cell cycle checkpoints activation

The term ‘cell-cycle checkpoint’ refers to mechanisms put in place to enforce dependency in the cell cycle by ensuring timely execution of process such as DNA replication or mitosis [73]. Thus, checkpoint pathways have the ability to control phase transitions. Given the critical significance of error-free DNA replication and chromosome segregation for the maintenance of genomic integrity and the prevention of cancer, cells have evolved the ability to trigger different cell cycle checkpoints upon DNA damage. These can transiently delay cell-cycle progression in G1, S or G2 phases or even impose prolonged cell-cycle arrests in either G1 or G2 before entry into the subsequent S phase or mitosis (Figure 7). At the molecular level, common denominator of all checkpoint pathways is the inhibition of cell cycle "controllers", namely the cyclin-dependent kinases. These are protein complexes responsible of ensuring the timely triggering and the execution of key events at phase transitions.

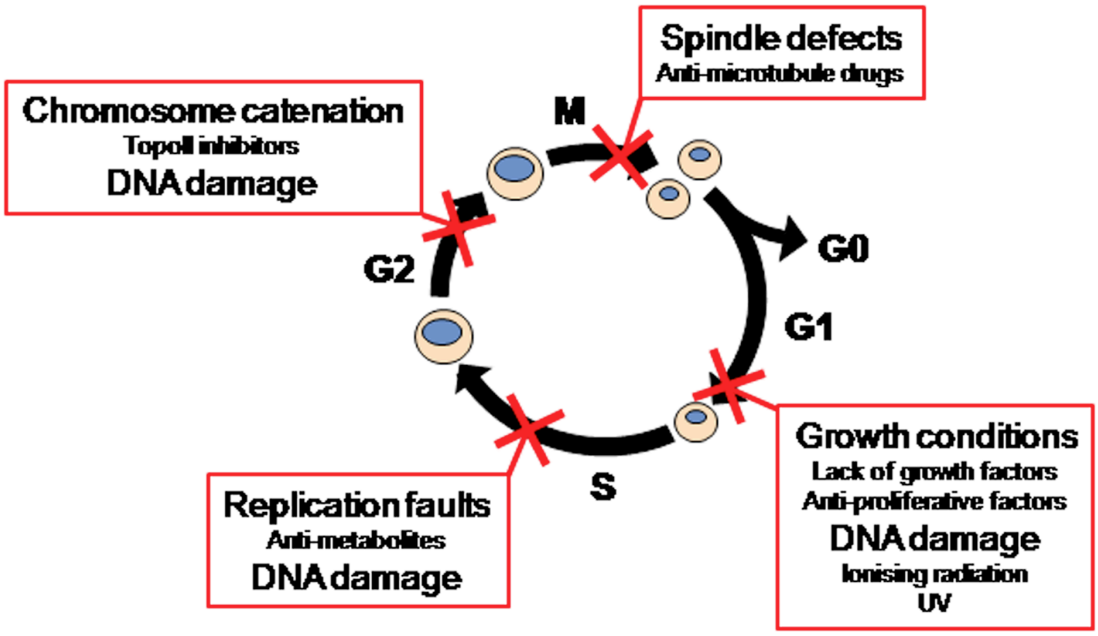

Figure 7. Cell cycle checkpoints. Modified after (Gabrielli, B., K. Brooks, and S. Pavey, Defective cell cycle checkpoints as targets for anti-cancer therapies. Front Pharmacol, 2012. **3**: p. 9.)

1.2.2.1 The G1/S checkpoint

To prevent replicating damaged DNA, cells exposed to genotoxic stress in G1 phase activate ATM/ATR. The latter, in a cascade of phosphorylation events, activate the downstream kinases CHK2/CHK1 that, in turn, directly phosphorylate the protein kinase WEE1. WEE1 phosphorylates CDK2 at two residues (Thr14/Tyr15) located in Gly-rich P-loop of the kinase, which is the ATP binding site. Such phosphorylation does not affect nucleotide binding but hampers catalysis [79]. In addition, checkpoint kinases phosphorylate members of the CDC25 family of double-specificity phosphatases on several serine or threonine residues. CDC25 phosphatases specifically remove phosphate from the two inhibitory sites in CDKs ATP-binding site, thus causing full activation of the CDK/Cyclin complexes [74]. Whereas Cdc25B and -C are not required for mouse development or checkpoint function [75], and inhibition of these phosphatases by DNA damage essentially occurs by sequestration mechanisms (see below), CDC25A degradation via ubiquitin-proteasome pathways is a primary control mechanism both in dividing cells and in response to DNA damage [76]. Phosphorylation of CDC25A at Ser-124 by CHK2 was reported to be the primary event responsible for ubiquitylation-dependent degradation of the phosphatase. Later work showed, however, the inconsistency of these observations [77]. Specifically, phosphorylation of CDC25A on Ser-76 by CHK1 was shown to serve as priming event that facilitates phosphorylation on Ser-79 and Ser-82 by protein kinase CK1 or glycogen synthase kinase-3β (GSK-3β) [78]. This, in turn, allows recruitment of the $SCF^{\beta\text{-}TRCP}$ E3 ligase that promotes CDC25A ubiquitylation [79]. The CHK1/CHK2-CDC25A checkpoint is rapid, since it is dependent on post-translational modifications, but it is also transient and can delay the G1/S transition only for a few hours. If the cell needs to impose a prolonged G1 arrest, a second pathway involving the tumour suppressor p53 is activated. Briefly, upon DNA damage p53 is directly phosphorylated by both ATM/ATR at Ser-15 and Ser-37 and the transducer kinases CHK2/CHK1 at Ser-20. In addition, the E3 ubiquitin ligase MDM2 that normally binds p53 and ensures rapid p53 turnover, is targeted by ATM/ATR, as well as by CHK2/CHK1 [80].

These simultaneous modifications of p53 and MDM2 lead to disruption of the dimeric complex, $p19^{ARF}$-mediated sequestration of MDM2 in the nucleolus with consecutive stabilization and accumulation of the p53 protein. Acetylation of p53 at the C-terminal Lysines that were target of MDM2 as well as at additional sites in the DNA binding domain facilitate p53 tetramerization, binding to its responsive elements in gene promoters and induction of transcription [81]. p53 most prominent targets at the G1/S transition is $p21^{CIP1/WAF1}$ (inhibitor of cyclin-dependent kinases). Specifically, $p21^{CIP1/WAF1}$ inhibits kinase activity by physical engaging the complex with an inhibitory domain that stretches over both CDK2 and Cyclin E to obstruct substrate binding [82]. Inhibition of CDK2/Cyclin E activity, in turn, results in incomplete phosphorylation of the tumor suppressor pRb, with consequent failure in releasing E2F1-3, the activating members of the E2F family of transcription factors, that are responsible for the transcription of Cyclin A and other S-phase genes [83] .

1.2.2.2 The S-phase checkpoint

During the S phase of cell cycle, human cells replicate the entire genome, which is composed of some three billion base pairs, to obtain two identical copies. In order to preserve the genetic information the duplication has to be carried out with highly fidelity. Cells encountering altered DNA structures during replication activate the so-called intra-S-phase checkpoint, which slows down ongoing DNA synthesis and prevents firing of new origins. This checkpoint operates via two parallel pathways, both of which are regulated by the ATM/ATR signaling cascade.

One branch of these effector mechanisms is through the CDC25A-degradation cascade described above. In addition to regulating CDK2/CyclinE, CDC25A is able to remove inhibitory phosphates from CDK2/CyclinA complexes to promote loading of the initiation factor CDC45 onto chromatin. CDC45 is a protein required for the recruitment of DNA polymerase α into assembled pre-replication complexes, hence the inhibition of CDK2 activity result in the inhibition of new origin firing [34].

The other branch of the intra-S-phase checkpoint operates through the ATM-mediated phosphorylation of NBS1 [75] and SMC1 (Structural maintenance of chromosomes 1) on several sites. However, how phosphorylated SMC1 contribute in slowing down DNA replication is unknown.
Both branches of the intra-S-phase checkpoint lead to a transient slow down of DNA replication to allow repairing the lesions before completion of replication. In case of failure to repair damage during this transient delay, cells complete replication and exit S phase. However, cells subsequently arrest in the G2 phase to tackle the persistent DNA damage.

1.2.2.3 The G2 checkpoint

The G2 checkpoint prevents cells from entering mitosis when they suffer DNA damage during transition through the G2 phase, or alternatively if they have progressed into G2 with unrepaired DNA lesions that occurred during the previous S or G1 phases. As sketched for the G1/S checkpoint, the final target of the G2 checkpoint is a CDK. Signals from unfinished DNA replication (through ATR/CHK1) or damaged DNA (through ATM/CHK2) activate the kinases WEE1/MYT1 that, in turn, phosphorylate active CDK1/CyclinB complexes causing inhibition of enzymatic activity. In parallel, checkpoint kinases phosphorylate CDC25 phosphatases [76]. Particularly, CHK1-dependent phosphorylation of CDC25A at Ser-124 and Thr-507 and of CDC25C at Ser-216 mediate recruitment of 14-3-3 proteins that displace the phosphatases from the nucleus, a mechanism that appears to be the primary way employed to inhibit CDC25A function during G_2 and mitosis [84] as well as CDC25C. The mechanism of inhibition of CDC25B, which mediates the activation of CDK1/Cyclin B at the centrosome during prophase, has been extensively studied in relation to its mitotic role [85] but is less characterized in response to DNA damage.
Moreover, additional factors upstream of CDC25 or cyclin B/CDK1, such as the Polo-like kinases PLK3 and PLK1 [86], the PLK1 activator AurA [87] and protein phosphatase PP2A [88] are also part of the G2/M checkpoint. Similar to G1 checkpoint, maintenance of the G2/M checkpoint partly relies on transcriptional regulation by p53 that, upon stabilization, induces transcription

of the cell-cycle inhibitor $p21^{CIP1/WAF1}$. In addition, the expressions of 14-3-3σ (scaffold and signaling protein), PUMA (BCL2 binding component 3), BAX (BCL2 partner and apoptotic activator), GADD45 (growth arrest and DNA-damage-inducible gene) are also regulated and are required for efficient arrest [86].

1.3 DNA nucleases

1.3.1 General features of DNA nucleases in DNA damage response

DNA, the carrier of genetic information of the majority of living organism, is composed of a sugar-phosphate backbone and four organic bases. DNA suffers from various environmental stresses, including attacks by UV light, radiation and carcinogens, which constantly modify its structure. Moreover, DNA accumulates errors that are intrinsic to the process of replication and displays unusual structures during recombination. In order to avoid alterations of the base sequence or entanglement of the DNA, these modifications must be corrected by the various repair protein machineries. Such DNA repair proteins usually form complexes with other proteins, likely to facilitate targeting and gain efficiency. A core component of these complexes are nucleases, which play crucial roles in recognizing and processing replication or recombination intermediates. In addition, through their participation to various DNA repair processes such as mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER) and double strand break repair, they also play a role in resolving DNA mismatches that occur during replication or eliminating damaged nucleotides.

As previously elucidated, double-strand breaks can be repaired by two main mechanisms depending on the phase of the cell cycle in which the lesion occurs, namely Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) [89]. The latter is based on the presence of the sister chromatid which is used as a template for the invading single-stranded DNA filament to elongate.

Such single-stranded DNA filaments are 3' overhangs generated during the process of resection, the initial step in the HR pathway to repair DSBs. Resection is performed by nucleolytic enzymes (Table 2) which can be regarded as molecular scissors that catalyzes the cleavage of phosphodiester bonds between the phosphate and the sugar moieties in the backbone of the DNA. Nucleases can be generally divided into exonucleases and endonucleases. Exonucleases can be further classified as 5'-end processing or 3'-end processing enzymes, according to the polarity of consecutive cleavage. On the other hand, endonucleases hydrolyze internal phosphodiester bonds within a polynucleotide chain, without the requirement of a free DNA end.

Summary of substrate specificities of selected nucleases and their role in various pathways.

Nuclease	Substrates for endo-activity	Substrates for exo-activity	Pathways
Mus81-EME1	3′-flap, nHJ, mHJ, fork, D-loop		RF restart, HR, ICLR, TM
XPF-ERCC1	3′-flap, bubble, D-loop, G-tail		NER, HR, ICLR, NHEJ, MMEJ, SSA, TM
SLX1-SLX4	5′-flap, HJ		HR, ICLR, SSA, RF restart, BER, NER, TM
GEN1	5′-flap, HJ		HR
FEN1	5′-flap, double flap	5′–3′ on nicked dsDNA	BER, OFM, TM
MRE11	5′-flap	3′–5′ on dsDNA	HR, NHEJ, MMEJ, TM
EXO1	5′-flap	5′–3′, 3′–5′ on dsDNA	HR, SSA, MMR, NER, OFM, TM
DNA2	5′-flap		OFM, HR, SSA, TM

RF restart – replication fork restart, HR – homologous recombination, ICLR – interstrand DNA crosslink repair, TM – telomere maintenance, NER – nucleotide excision repair, NHEJ – non-homologous end joining, SSA – single strand annealing, BER – base excision repair, MMEJ – microhomology mediated end joining, OFM – Okazaki fragment maturation.

Table 2. *Modified after (Nucleases in homologous recombination as targets for cancer therapy)*

Single-stranded 3'-overhanging DNA filaments represent an important substrate for many downstream events occurring during the repair of the lesion, such as Rad51 binding to initiate homologous pairing and strand exchange and Rad52-mediated annealing [90], activation of the DNA damage response and arrest of the cell cycle upon DNA damage [70]. Moreover, checkpoint proteins recognize ssDNA and also affect the rate at which ssDNA arises, suggesting that the DDR regulates accumulation of ssDNA by regulating the activity of nucleases [91]. Mutations in some of the nucleases involved in the DDR are directly associated with diseases or they show defects in DNA repair, accumulation of damage and greater risk of genome instability leading to predisposition to cancer (Table 3). Furthermore, DNA repair enzymes, thus including nucleases, are also commonly upregulated in some cancer types to ensure efficient repair of DNA damage caused by chemo- or radiotherapy, and this can lead to drug resistance. Interfering with

nucleases activity by chemical inhibition or downregulation may potentiate the effects of anti-cancer drugs and it may represent a new approach in the synthetic lethality therapy [92].

Expression levels of selected HR factors identified in primary cancers.

Nuclease	Increased expression	Decreased expression
MUS81		Gastric, colorectal, hepatic cancer
ERCC1	Ovarian, colorectal, gastric, head and neck, non-small-cell lung cancer	Gastric, colorectal, non-small-cell lung cancer
FEN1	Testes, lung, brain, breast, kidney, pancreatic, colon cancer	Lung, prostate, gastrointestinal cancer
MRE11		Ovarian, breast, colorectal cancer
RAD50		breast cancer
NBS1	Neck and head cancer, melanoma	Breast, colorectal cancer, melanoma
EXO1	Atypical HNPCC	
DNA2	Breast, pancreatic, ovarian cancer, pancreatic ductal adenocarcinoma	
RAD51	Breast, pancreatic, head and neck, non-small-cell lung cancer	Colorectal, breast cancer
RAD52		Colorectal cancer

Expression confirmed by mRNA/protein level measurements from cancer patients.

Table 3. *Modified after (Nucleases in homologous recombination as targets for cancer therapy)*

Synthetic lethality or sickness (SLS) is the most promising approach for cancer therapy; it requires two mutations which, by themselves, are not effective on cell survival, but when combined they lead to cell death. If any of the two genes is cancer-specific, upon inhibition of the second gene it results in higher selective killing of cancer cells without side toxic effects on normal cells. Since cancer cells are often defective for DDR factors and DNA repair proteins, the use of specific repair inhibitors will take advantage of this concept. Indeed, breast cancer patients defective for the recombination mediator BRCA2 and treated with chemical inhibitor against the base excision repair factor Poly (ADP-ribose) polymerase (PARP), show increases sensitization of cancer cells due to the interference with the ssDNA breaks repair pathway by the PARP inhibitor [93, 94].

In this case, replication forks are stalled and ssDNA nicks are converted into dsDNA breaks that cannot be repaired in these recombination-deficient cells. HR-deficient cancer cells are also induced to higher sensitivity upon PARP inhibition due to the same concept. [95, 96]. Regarding nuclease involved in HR, some examples are provided: ERCC1-defective cells resulted to be synthetically lethal with ATR inhibition. ERCC1 represent the non-catalytic

subunit of XPF protein which is an endonuclease involved in many DNA repair pathways such as nucleotide excision repair (NER), interstrand crosslink repair (ICLR), HR and NHEJ [97]. CDC4- and MRE11-defective cancer cells [98] and RAD54B-defective colorectal cancer cells [99] have shown synthetic lethality upon FEN1 inhibition; FEN1 is a member of the Rad2/XPG family of exonucleases and it has also gap endonuclease and RNaseH activities. It has a very important role in DNA replication and repair [100]. Finally BLM helicase-deficient cells depleted for MUS81 complexes are synthetic lethal. MUS81 is another endonuclease of the XFP family which acts in complex with the non-catalytic subunits EME1 and EME2; it has increased expression during S/G2 transition of the cell cycle which confirm its role in processing replication and recombination intermediates. Indeed MUS81 is responsible for the cleavage of collapsed replication forks induced by DNA damaging events [101, 102].

1.3.1.1 Exonuclease 1 (EXO1)

EXO1 is a member of the Rad2/XPG family of nucleases. It was originally identified in the fission yeast *Schizosaccharomyces pombe* upon induction to undergo meiosis. *S. pombe* Exo1 was shown to catalyze the removal of mononucleotides from the 5' end of a DNA duplex or DNA nicks by degrading in 5'-3' polarity, acting preferentially on double-stranded DNA ends (dsDNA) producing 3' single stranded overhangs. The human EXO1 gene encodes a protein that bears only 27% identity to its yeast ortholog [103]. However, it has been demonstrated that human EXO1 can complement the DNA damage sensitivity and the mutator phenotype that result from deletion of Exo1 in *Saccharomyces cerevisiae* [104], indicating that at least certain aspects of EXO1 function appear to be conserved. In vitro, human EXO1 was show to be a structure specific nuclease, possessing 5'-3' exonuclease and 5'-flap endonuclease activities [105].

EXO1 has also been reported to possess RNase H activity. EXO1 molecular mechanism of action was recently clarified and shown to be similar to that of other FEN nucleases [106].

EXO1 is involved in several DNA repair pathways including MMR, double strand break repair, post-replication repair as well as meiotic and mitotic recombination [107] and telomere maintenance. The first established role for yeast EXO1 was deduced from its ability to physically interact with yeast and human MSH2, followed by the demonstration of its participation in MMR [108]. It was later demonstrated that EXO1 plays both catalytic and structural roles during MMR-mediated repair [109]. Briefly, upon detection of a mismatched base, the MMR machinery is recruited (Figure 8), EXO1 carries out a controlled 5' to 3' directed excision step that removes nucleotides in the newly made strand past the mismatch, creating a ssDNA gap that serves as a platform for DNA polymerase. The polymerase fills in the excised stretch, finally a DNA ligase seals the nick completing the MMR [110].

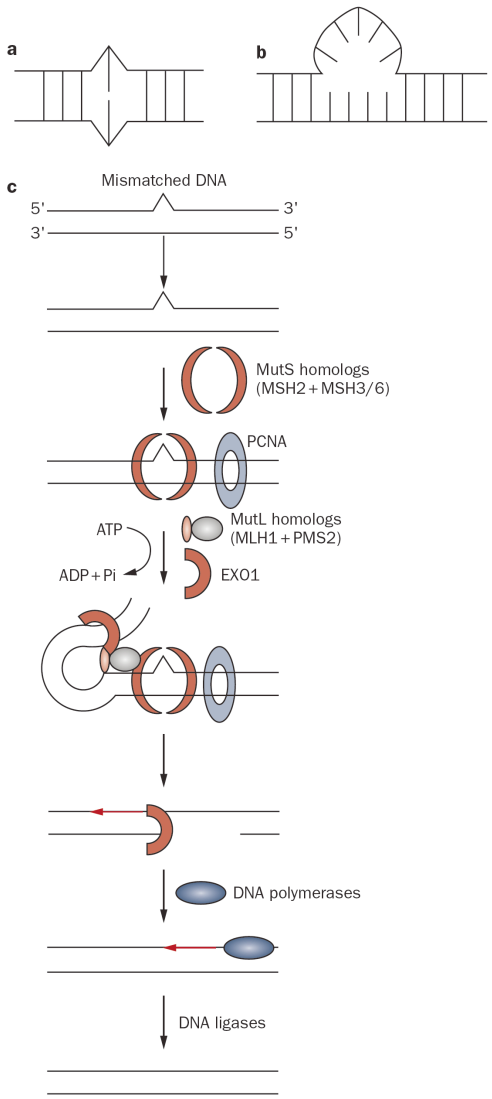

Figure 8. Schematic representation of mismatches and MMR pathway involving EXO1 activity. The MMR system recognizes base-base mismatches (a) or an insertion-deletion loop (b). MutS homologs bind to the affected site of DNA, which triggers ATP-dependent conformational changes and the binding of MutL homologs. These in turn recruit other proteins, including PCNA and EXO1 with the subsequent excision of the damaged strand. The interactions of the bound proteins trigger DNA looping, which brings the two sites together. The resultant gap in the strand is then filled by DNA polymerases and the break is removed by DNA ligase Modified after (Hewish, M., et al., *Mismatch repair deficient colorectal cancer in the era of personalized treatment.* Nat Rev Clin Oncol, 2010. **7**(4): p. 197-208).

The involvement of EXO1 in DNA repair pathways suggests it could be also be a target for mutation in tumourigenesis. Consistent with this, a cancer-prone phenotype has been observed in Exo1-defiecient mice, where a clearly increased susceptibility to lymphoma development was observed [111]. Mounting evidence has shown that EXO1 plays an important role in DSB repair by executing a long-range resection step to generate extended stretches of ssDNA. This ssDNA serves to induce cell cycle checkpoints and is required for RAD51 mediated strand invasion of the sister chromatid for an efficient HR cascade. Cells depleted of EXO1 show chromosomal instability, hypersensitivity to IR and defects in HR-dependent DSB repair [112]. In a two-step mechanism of DSB repair, the MRN complex detects a DSB and, collaborating with CtIP, promotes DNA end resection generating short ssDNA overhangs [44]. This initial resection step is followed by a more extensive step of resection carried out by redundant enzymes with EXO1 being an important exonuclease among them [113].

Accumulating evidence suggests that EXO1 plays also an important role at the replication fork. Data from *Saccharomyces cerevisiae* demonstrated that Exo1 acts in a redundant manner with Rad27 (FEN1 in human) in processing Okazaki fragments during the process of DNA replication [111]. Moreover, Exo1 was shown to be recruited to stalled replication forks where it plays a role in preventing fork reversal by resecting newly synthesized strands and helping to resolve sister chromatid junctions [83]. During NER, EXO1 promotes enlargement of DNA gaps, which is a signal for ATR-mediated DNA damage response [114-116].

EXO1 nucleolytic activity is tightly regulated under DNA replication stress and other cellular responses to DNA damage. It was demonstrated in our and other laboratories that such control is exerted either by post-translational modifications or by direct interaction with other proteins. Results from our laboratory show that in mammalian cells, upon replication fork stalling, EXO1 is phosphorylated in an ATR-dependent manner, with this phosphorylation targeting it to ubiquitin-mediated degradation through the proteasome pathway. We also showed that EXO1 interacts with CtIP *in vivo* and *in vitro* and that its exonucleolytic activity is restrained *in vitro* by this interaction [117]. Others have shown that also ATM-mediated signals can control EXO1, namely by providing evidence that phosphorylation of EXO1 by ATM occurs in response to ionizing radiation and result in a decrease of enzymatic activity, in a manner that allows loading RAD51 and the completion of HR. At telomeres, EXO1 exerts extensive G-rich strands resection promoting telomeric recombination, which leads to increase genomic instability in telomerase-deficient cells [118].

Missense mutations in EXO1 have been identified in patients with atypical HNPCC (non-polyposis colorectal cancer) [119] and EXO1-deficient mice show reduced survival, higher mutation rates and increased susceptibility to develop lymphomas [111]. A multi-mutations hypothesis was suggested by in-vitro studies on yeasts, in which a weaker mutation phenotype of EXO1 might be combined with other mutator alleles acquiring pathogenic features [120]. On the other hand, HNPCC patients over-expressing EXO1 show increased genetic instability and promoted cancer progression probably due to the loss of the mutant allele [133].

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2. REVIEW

It takes two to tango: Ubiquitin and SUMO in the DNA damage response

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It takes two to tango: Ubiquitin and SUMO in the DNA damage response

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Abstract

The complexity of living cells is primarily determined by the genetic information encoded in DNA and gets fully disclosed upon translation. A major determinant of complexity is the reversible post-translational modification (PTM) of proteins, which generates variants displaying distinct biological properties such as subcellular localization, enzymatic activity and the ability to assemble in complexes. Decades of work on phosphorylation have unambiguously proven this concept. In recent years, the covalent attachment of Ubiquitin or Small Ubiquitin-like modifiers (SUMO) to amino acid residues of target proteins has been recognized as another crucial PTM, re-directing protein fate and protein-protein interactions.

This review focuses on the role of ubiquitylation and sumoylation in the control of DNA damage response (DDR) proteins. To lay the ground, we begin with a description of ubiquitylation and sumoylation, providing established examples of DNA damage response elements that are controlled through these PTMs. We then examine in detail the role of PTMs in the cellular response to DNA double-strand breaks illustrating hierarchy, cross-talk, synergism or antagonism between phosphorylation, ubiquitylation and sumoylation. We conclude offering a perspective on ubiquitin and SUMO pathways as targets in cancer therapy.

Introduction

The components of signal transduction pathways are organized in a hierarchical manner and communicate with one another. In its simplest formulation, a signaling pathway can be represented with a linear cascade where unidirectional arrows connect a stimulus to the final response through a defined number of intermediates. The recent sequencing of animal and plant genomes and the advent of systems biology have changed this perspective. Proteome scale interaction studies have unveiled the existence of interfaces between pathways and shown that the multiplicity of interactions among their components likely accounts for the arrays of biological outputs observed. While this novel perspective represented *per sè* a step forward, it still had the intrinsic limitation of merely providing a static snapshot of biological networks. The need for a more realistic picture of signal transduction prompted the development of predictive modeling that, by representing the dynamic flow of information, accounts for the fluctuation of variables as it actually occurs in defined biological systems (Barabasi and Oltvai, 2004). Despite their intrinsic limitation though, “snapshots” provided by reductionist approaches currently represent our best option to study and explain the functioning of signal transduction networks at the molecular level.

Considering that proteins are the constitutive elements of cellular networks and they hierarchically relate to each other, modification of structural or enzymatic traits of one or more elements in a network will necessary affect the network properties and result in outputs that are directly observable (i.e., cell proliferation in response to growth factors, cell cycle arrest or terminal differentiation in response to antimitogens or differentiation factors, respectively). Alteration of the properties of network components is achieved through post-translational modifications (PTM), consisting in the covalent addition of chemical groups to one or more amino acids of a protein target in a manner that is, in most cases, reversible. The hierarchical, synergistic or antagonistic combination of PTMs defines a code that translates in distinct outputs.

Historical perspective

Ubiquitin entered the arena of scientific discoveries in 1978 as result of pioneering work initiated in the midst of more trendy studies addressing how the information contained in DNA is decoded to generate the variety of proteins that make up a cell (Ciechanover, 2009). The concept prompting this pioneering investigation was that synthesis and destruction of cellular proteins are homeostatic, with a perfect equilibrium being a necessary condition for life. Ciechanover and colleagues observed that reticulocytes get rid of lysosomes during terminal differentiation but retain the ability of degrading hemoglobin. Starting from this observation, they set out to identify this non-lysosomal mechanism of protein degradation. Using classic biochemical protocols consisting of chromatographic fractionation of crude cell extracts followed by reconstitution of the enzymatic activity of interest through complementation of fractions, they discovered that proteolysis occurs through a cascade of events culminating in the covalent addition of a heat-stable component to proteins targets. Such component was named ATP-dependent proteolysis factor 1 (APF-1) and is now known as ubiquitin (Ciehanover et al., 1978). Protein modification by APF-1, in turn, was shown to facilitate selective target recognition by the proteolytic machinery (Hershko et al., 1980). The subsequent discovery of several ubiquitin-like proteins (UBLs) helped shedding light on the complexity of this post-translational modification. UBLs were essentially demonstrated to have functions other than the control of protein degradation. This is the case of NEDD8, which can be covalently linked to cullins (Hori et al., 1999), the scaffold components of multisubunit ubiquitin E3-ligases, affecting their activity; ISG15, which is attached to components of signal transduction pathways triggered by IFN-γ or lipopolysaccharide and plays a role in inflammatory and immune responses (Malakhov et al., 2003); Urm1, which has low sequence homology to ubiquitin (Goehring et al., 2003), though it displays a similar fold and is involved oxidative stress responses in yeast; finally, the Atg cascade controlling autophagy in yeast and man, which is the main mechanism responsible for the degradation of cellular components in response to nutrients starvation. This consists of the E1-like enzyme Atg7, the E2-like components Atg3 and

Atg7, and the E3-like Atg12-Atg5 conjugate that facilitates transfer of the ubiquitin-like modifier Atg8 to phospholipids (Hanada et al., 2007).

Ubiquitylation

Ubiquitin is a highly conserved regulatory protein of 76 amino acids (8.5 kDa), which is constitutively expressed in all tissues of eukaryotic organisms. In mammalian cells, ubiquitin is encoded by 4 genes: RSP27A, UBA52, UBB and UBC (Kimura and Tanaka, 2010). The ATP-dependent conjugation of ubiquitin C-terminal glycine (G_{76}) to lysine residues in the substrate leads to the formation of an isopeptide bond. Ubiquitin itself contains 7 lysines behaving as acceptors for additional ubiquitin molecules to generate poly-chains. Ubiquitylation is carried out in a cascade of reactions: first, a thiolester bond is formed in an ATP-dependent manner between a cysteine in the active site of the E1-activating enzyme and ubiquitin G_{76}. Second, ubiquitin is transferred to the active cysteine of an E2-conjugating enzyme. Finally, an E3-ligase enzyme binds the E2-Ub complex and transfers ubiquitin to lysine residues of the acceptor substrate (Hershko and Ciechanover, 1998) (Fig. 1). Mammalian cells express only 2 E1s, approximately 38 E2s and more than 600 E3s.

E2-conjugating enzymes - E2-conjugting enzymes can be classified in 17 subfamilies (Michelle et al., 2009) characterized by an active core called UBC (ubiquitin-conjugating) domain that contains the cysteine residue required for catalysis. Five of the 38 known E2 enzymes lack the catalytic cysteine and are called UEV (ubiquitin E2 variant) proteins. UEV can either bind an active E2, thus regulating its activity, or bind an activating cofactor once they are ubiquitylated (Polo et al., 2002). Ubiquitin E2 enzymes are structurally similar to UBL modifiers E2s, though the former can specifically interact with the two E1s involved in ubiquitylation (Ye and Rape, 2009). Each E2 enzyme can interact with multiple E3s, as demonstrated for Cdc34 (E2) and SCF complexes (E3) (Skowyra et al., 1997) or the UBE2C / UBE2S (E2s) and the APC/C (anaphase promoting complex / cyclosome; E3) (Williamson et al., 2009) or as shown in network interaction studies (Markson et al., 2009). Specificity is provided by the N-terminal region of the E2 where the amino

acidic sequence determines the secondary structure of loops (L1 and L2) specifically contacting two loops and an α-helix of the E3 (Zheng et al., 2000). For E2s interacting with more than one E3, the residue involved in recognition usually differs from one E3 to the other (Zhang et al., 2005a). The binding affinity between ubiquitin-charged E2s and their cognate E3s is generally high, rendering very fast the kinetic of interaction (Das et al., 2009). Moreover, binding sites for E1 and the specific E3 often overlap in the E2, such that the E2 must dissociate from the E3 to be charged with ubiquitin by the E1 and vice-versa (Eletr et al., 2005). E2 enzymes catalyze ubiquitin chains initiation and elongation. Whereas some of them, such as UBE2W and UBE2E in humans, are specifically used by their E3, BRCA1, for chain initiation, the heterodimeric complex UBE2N-UBE2V1 and UBE2K are mainly involved in chain elongation (Christensen et al., 2007;Rodrigo-Brenni and Morgan, 2007;Jin et al., 2008b). Few E2s can mediate both processes, as illustrated by yeast Cdc34 that, together with SCF, is responsible for initiating ubiquitin chains formation on Sic1 (cell cycle inhibitor subunit of cyclin-dependent kinase 1) in a non-interacting manner, but also for the chains elongation by direct interaction with the substrate (Petroski and Deshaies, 2005).

E3-ligases - E3s are often part of multimeric complexes and can be divided in two main classes: HECT (Homologous to E6AP COOH-terminus) and RING (Really Interesting New Gene). HECT E3s bind ubiquitin on a cysteine in their catalytic domain and transfer it to the substrate in an E2-independent manner (Kulathu and Komander, 2012). The C-terminal domain of HECT E3s is highly conserved and retains both catalytic activity and the determinants for chain type specificity (You and Pickart, 2001), while the N-terminal region determines substrate specificity (Huang et al., 1999). Established members of the HECT family are E6AP, a partner of the oncogenic E6 protein of human papillomavirus, responsible for p53 downregulation (Huang et al., 1999), Itch/AIP4, with roles in the inflammatory signaling pathways (Chastagner et al., 2006) and Nedd4 and Nedd4L that participate in the development of mouse central nervous system (Kumar et al., 1992).

The vast majority of E3-ligases known to date belongs to the RING family and

is characterized by the presence of the Cys/His-rich RING finger domain. The RING finger brings in close proximity substrate and activated E2 enzyme, with the latter directly transferring ubiquitin to the former. A domain structurally related to the RING finger, the U-box, is found in many E3 ligases of this class (Deshaies and Joazeiro, 2009). Rad18 was the first identified RING domain-containing protein and, together with the E2-conjugating enzyme Rad6, was shown to be essential for post-replicative bypass of UV-induced DNA damage in yeast (Bailly et al., 1997). The RING domain, along with a B-box (zinc-binding fold similar to the RING) and a coiled-coil region (CC), collectively called RBCC supradomain, characterizes the 80 members of the TRIM (Tripartite Interaction Motif) family of E3-ligases (Marin, 2012). A small subfamily of E3 ligases is characterized by the presence of three RING domains: an N-terminal (N-RING), a in-between (IBR) and a C-terminal (RING2 or C-RING) (Eisenhaber et al., 2007). Parkin, a protein involved in Parkinson's disease, is the best characterized member of this subfamily (Chaugule et al., 2011). The Cullin/RING ubiquitin ligase (CRL) subfamily represents the largest subgroup of the RING-finger E3 ligases (Deshaies and Joazeiro, 2009). CRLs are multisubunit E3s composed of a RING finger domain protein (Rbx1 or Rbx2) responsible for recruiting the ubiquitylated E2 enzyme, a scaffold protein member of the Cullin family and a receptor for substrate recognition (F-box protein). Some CRLs additionally feature a linker protein, such as Skp1 in the SCF complex or CRL1 and DDB1 in the CRL4 complex (Deshaies and Joazeiro, 2009). CRLs are activated by a post-translational modification consisting in conjugation of NEDD8 to the Cullin component (Pan et al., 2004).

Atypical ubiquitin chains - Ubiquitylation indicates the process of single ubiquitin moiety addition to a substrate as well as its extension to form ubiquitin polymers. Chain extension can occur at all seven lysine residues present on ubiquitin (K_6, K_{11}, K_{27}, K_{29}, K_{33}, K_{48}, K_{63}) (Ikeda and Dikic, 2008). E2s such as UBE2N (UBC13) or UBE2RI (CDC34) show specificity for linkage to K_{63} or K_{48}, respectively (VanDemark et al., 2001;Petroski and Deshaies, 2005). Others, like UBE2D and UBE2E, can promote different types of ubiquitin chains formation (Kim et al., 2007). K_{48} and K_{63} linked

chains represent the two mostly studied modifications by ubiquitin, with the first essentially involved in degradation by the 26S proteasome (Komander and Rape, 2012) and the second mainly affecting the function of signaling components (Sun and Chen, 2004) and DNA repair proteins (Chen and Sun, 2009). Proteins undergoing degradation are recognized by the substrate receptor component of the 26S proteasome only if they contain chains longer than 4 ubiquitin moieties (Thrower et al., 2000). The processivity of such chains, which is the number of ubiquitin moieties attached to a protein or to a growing ubiquitin chain while it is associated to the E3 ligase, determines the rate of substrate degradation (Rape et al., 2006). K_6-linked chains do not likely have a proteolytic role (Kim et al., 2011). K_{11}-linkage, on the contrary, plays a key role in the degradation of cell cycle regulators as well as in endoplasmic reticulum associated degradation (ERAD) and membrane trafficking (Behrends and Harper, 2011). Little is know about the relatively low abundant K_{27}-, K_{29}- and K_{33}-linkages (Komander and Rape, 2012). An additional type of ubiquitin chain assembly was recently discovered, the so-called LUBAC (linear ubiquitin chains assembly complex), which is formed by a complex of two E3 RING-finger ligases, HOIL-1L and HOIP. This type of linkage is characterized by head-to-tail assembly, in which the C-terminal glycine of the previous ubiquitin is linked to the methionine residue of the next ubiquitin. Linearly-linked ubiquitin chains are mainly involved in targeting proteins with a role in innate and adaptive immune signaling pathways (Walczak et al., 2012).

Deubiquitylating enzymes – Ubiquitylation is a reversible process, with deubiquitinases (DUBs) being responsible for the disassembly of ubiquitin chains (Nijman et al., 2005). Deubiquitylation controls cell cycle transitions, proteasome- and lysosome-dependent degradation pathways, DNA repair, endocytosis and signal transduction pathways among others. Importantly, DUBs participate in controlling the dynamic state of histone ubiquitylation. An essential function played by DUBs is the co-translational activation of ubiquitin, which is expressed as fusion to ribosomal proteins or in linear poly-ubiquitin chains (Reyes-Turcu et al., 2009). A second important function is the recycling of free ubiquitin from unattached chains (Komander et al., 2009).

The human genome encodes approximately 100 DUBs, distinguished in five families: ubiquitin C-terminal hydrolases (UCH), ubiquitin specific proteases (USP/UBP), ovarian tumor (OUT), Josephines and JAB1/MPN/Mov34 metalloenzymes (JAMM) (Reyes-Turcu et al., 2009). Whereas the first four families behave as cysteine proteases, JAMM function as zinc-dependent metalloproteases. To prevent inappropriate or unscheduled cleavage of substrates, DUBs activity is controlled by a variety of PTMs, including phosphorylation, ubiquitylation and sumoylation (Reyes-Turcu et al., 2009). Besides the catalytic domain, DUBs feature protein-protein interaction domains and ubiquitin-binding domains that facilitate formation of multimeric complexes and interaction with substrates, respectively. In most cases, binding to ubiquitin causes DUBs to undergo conformational changes that expose the catalytic site, which is often hidden by a loop or a larger domain (Reyes-Turcu et al., 2009). DUBs such as USP14, UCH37 and POH1 are often found associated with the 19S subunit of the proteasome, a feature that allows hydrolyzing the poly-ubiquitin chain from the substrate and recycling ubiquitin prior to channeling the target protein into the proteasome (Finley, 2009). Reactive oxygen species (ROS) reversibly inactivate Cys-based DUBs, as exemplified by the key regulator of genomic stability USP1, the oxidation of which facilitates PCNA mono-ubiquitylation and the consecutive recruitment of Polη for the repair of oxidation-induced lesions (Cotto-Rios et al., 2012).

Shuttling to the proteasome - The destiny of proteins modified by K_{48} poly-ubiquitin chains is degradation by the 26S proteasome. In the DNA damage response (DDR), this task is facilitated by shuttling that is orchestrated by dedicated receptor proteins such as yeast Rad23, Dsk2, Ddi1 and the Shp1/Cdc48/p97 complex. Receptor proteins recognize poly-ubiquitin chains in their targets by virtue of Ubiquitin-Associated (UBA) domains and interact with subunits of the proteasome via Ubiquitin-Like (UBL) folds, thus effectively shuttling cargoes to the proteasome (Grabbe and Dikic, 2009). The yeast Rad23, which was originally identified for its role in nucleotide excision repair (NER), and its human homologues hHR23A and hHR23B are paradigmatic to this pathway. Rad23 contains two UBA and an N-terminal UBL domain that

dynamically interacts with either one of the two UBA domains (Goh et al., 2008). Binding of an UBA domain to poly-ubiquitin chains of the cargo protein displaces the UBL domain that becomes available for interacting with the proteosomal subunit 5a (Mueller and Feigon, 2003), facilitating the delivery of cargos to the proteasome. Paradigmatic is human p97 and its ubiquitin-binding partner, the heterodimer UFD1-NPL4, that are recruited to DNA lesions and selectively remove K_{48}-ubiquitin conjugates allowing the subsequent deposition of 53BP1, BRCA1 and Rad51 to regions undergoing repair (Meerang et al., 2011).

Sumoylation

SUMO proteins and ubiquitin have only limited sequence identity but they fold in a similar manner (Bayer et al., 1998). SUMO-2 and SUMO-3 are 95% identical but display only 43% identity to SUMO-1. SUMO proteins are generated as inactive precursors and processed by Sentrin/SUMO-specific proteases (SENPs) that catalyze the removal of a C-terminal oligopeptide, exposing the glycine that is conjugated to lysine residues in the target (Xu and Au, 2005).

As for ubiquitin, SUMO-1, SUMO-2 and SUMO-3 are conjugated to substrates through a dedicated E1-E2-E3 cascade. SUMO proteins bind the activating enzyme E1 (SAE1 and SAE2 in mammals, (Gong et al., 1999)) in an ATP-dependent manner and are transferred to the conjugating enzyme UBC9, which is the only E2 dedicated to SUMO conjugation (Johnson and Blobel, 1997). UBC9 is able to recognize and transfer SUMO to targets in the absence of a co-adjuvating E3, though E3-like proteins containing an SP-RING domain facilitate the process by enhancing the affinity of UBC9 for its substrates (Bernier-Villamor et al., 2002). In the absence of an E3, acetylation apparently provides a means for UBC9 to discern between substrates carrying extended vs. regular recognition motifs (see below) (Hsieh et al., 2013).

The distinct mechanism of SUMO recognition and conjugation likely depends on the different distribution of charged residues on the surface of SUMO proteins as compared to ubiquitin (Melchior, 2000). Of the SUMO E3-ligases identified to date, some display exquisite specificity, such as RanBP2 that

selectively targets RanGAP1 and Sp100 (Pichler et al., 2002). Others, like the PIAS family of proteins that are the mammalian homologues of yeast Siz proteins, act as repressors of STAT3 (Chung et al., 1997) and a number of transcription factors (Schmidt and Muller, 2003). Similarly to RING ubiquitin ligases, the Siz/PIAS SUMO E3-ligases do not physically bind SUMO but rather interact non-covalently with it. Furthermore, through their zinc-binding SP-RING domain they associate with UBC9. In this manner Siz/PIAS bring SUMO-loaded UBC9 in close proximity with the protein target and facilitate transfer of the SUMO mojety (Hochstrasser, 2001). Among other SP-RING type SUMO E3s, TOPORS was the first reported example of an E3 ligase supporting the transfer of both ubiquitin and SUMO (Rajendra et al., 2004;Weger et al., 2005).

SUMO-2 and SUMO-3 can polymerize to form chains on protein substrates whereas SUMO-1 is only added as monomer (Tatham et al., 2001). It is established that some substrates are modified either by SUMO1, namely RanGAP1 (Saitoh and Hinchey, 2000), or SUMO2/3, namely PML, whereas others are modified indifferently by both SUMO1 and SUMO2/3 (Vertegaal et al., 2006). The reason for such heterogeneity in the SUMO conjugation process is currently unknown, though it may be in part explained by the different pools of SUMO proteins available in the cell, with SUMO1 being mostly conjugated and SUMO2/3 forming a free pool that is mobilized in response to environmental stress (Saitoh and Hinchey, 2000).

The minimal core consensus sequence for recognition and sumoylation of target proteins is defined as Φ-K-X-D/E (with Φ being a hydrophobic residue). An extended sumoylation motif consisting in the sequence Φ-K-X-D/E-X_2-$(E/D)_{4-5}$ may comprise sites of phosphorylation in the acidic stretch that follows the sumoylated lysine (Yang et al., 2006).

As for other PTMs, sumoylation is a reversible process. The enzymes reversing sumoylation belong to the class of SENP proteins that control SUMO maturation from precursor polypeptides. Of the six SENP enzymes present in the mammalian genome, SENP1 and SENP2 display the ability of their yeast counterpart Ulp1 to control both the maturation of SUMO proteins and desumoylation reactions. SENP1 and SENP2 display a slight preference

for pre-SUMO1 or pre-SUMO2/3, respectively, in the process of maturation but act equally well on both during deconjugation (Xu and Au, 2005). SENP3 and SENP5 preferentially remove monomeric SUMO2/3 moieties, whereas SENP6 and SENP7 selectively act on SUMO2/3 chains and do not participate in the maturation of SUMO proteins (Mikolajczyk et al., 2007). SENP enzymes are themselves controlled by sumoylation, ubiquitylation and subcellular localization (Hickey et al., 2012).

The role of sumoylation at the organism level became apparent thanks to studies that were first conducted in budding yeast. These showed that depletion of Ubc9 causes cell cycle arrest at G2/M, with cells displaying large buds and containing a single nucleus and a short spindle (Seufert et al., 1995). Likewise, studies conducted in fission yeast showed that deletion of the Ubc9 homologue *hus5* is not lethal but results in chromosome segregation defects (al-Khodairy et al., 1995). Subsequent data from chicken DT-40 cells showed that Ubc9 is essential for the viability of higher eukaryotic cells and knockout results in the formation of multiple nuclei, likely due to cytokinesis defects, with a significant proportion of cells entering apoptosis (Hayashi et al., 2002). Studies conducted in mice confirmed the severe phenotype of Ubc9 knockout, with embryonic lethality observed at early post-implantation stage. Furthermore, blastocysts failed to expand after 2 days in culture and displayed defects in chromosome condensation and segregation as well as dismorphic nuclear envelopes and disruption of nucleoli and PML bodies (Nacerddine et al., 2005). Sumoylation has also been linked to human pathologies, in that human SUMO1 haploinsufficiency was found to be responsible for cleft lip and palate, a finding corroborated by a mouse model (Alkuraya et al., 2006). Others, however, reported no obvious developmental defects in SUMO1 knockout mice (Evdokimov et al., 2008;Zhang et al., 2008), suggesting possible redundancy among SUMO proteins.

A peculiarity distinguishing SUMO from other PTMs is the ability of triggering fully-fledged responses despite minor amounts of the proteins involved in the response are actually modified by SUMO, a phenomenon denoted as “the SUMO enigma” (Hay, 2005). This was demonstrated to be the case for transcriptional repression, where modification by SUMO is apparently required for the recruitment of transcription factors into repressive protein complexes,

with their sequestration remaining permanent even upon SUMO removal (Wilkinson and Henley, 2010). SUMO modification of only a small population of a substrate at any given time point was also suggested to occur for DNA repair proteins such as TDG. Thymidine-DNA glycosylase is part of the base excision repair system (BER) and displays the ability of specifically addressing uracil/thymidine base mismatches (Sancar et al., 2004). The rate-limiting step in the enzymatic reaction carried out by TDG is its dissociation from the abasic site (AP site) generated as first step in the BER process. The high affinity of TDG for the structure generated upon removal of the base is an important self-protection mechanism put in place by the cell since AP sites can turn into DNA strand breaks, thus threatening genome stability (Hardeland et al., 2002). Sumoylation is the appropriate solution to this issue, in that SUMO-modified TDG looses affinity for the abasic site allowing recruitment of the (AP)-endonuclease that acts in the next step of BER (Sancar et al., 2004). To re-initiate the circle, de-sumoylation by SENPs/ULPs renders TDG promptly available for the next round of lesion recognition and processing (Hardeland et al., 2002). Thus, SUMO modification of minimal amounts of TDG is sufficient to address the repair of uracil/thymidine base mismatches in a highly controlled manner.

The assembly of proteins complexes in response to sumoylation was addressed by means of two-hybrid screens that led to the discovery of proteins bearing SUMO-interacting motifs (SIMs) (Hannich et al., 2005). SIM-containing proteins display the ability to recognize a structure formed by the opposing β2-strand and α1-helix of SUMO1-3 that contain hydrophobic and positively charged residues and form a groove in which the SIM peptide can be bound in parallel or anti-parallel orientation with respect to the β2-strand of SUMO (Kerscher, 2007). Negative charges in the SIM-peptide, represented by acidic amino acids or phosphorylation sites, facilitate electrostatic interaction with lysines or arginines of SUMO β2-strand and α1-helix (Kerscher, 2007).

PTMs in DNA damage response: the old and the new

The cascade of events resulting from detection of DNA damage and

orchestrating its repair has been best described for DNA double-strand breaks (DSBs). A detailed account of ubiquitylation and sumoylation events occurring at DSBs will be followed by a brief mention to the signaling triggered by other types of DNA lesions.

DSB recognition - Initial players consist of proteins or proteins complexes such as Ku70 and Ku80 or MRE11/RAD50/NBS1 (MRN) that, through recognition and binding to DNA ends, facilitate recruitment and activation of the protein kinases DNA-PKcs or ATM, respectively. The latter function as transducers of the DNA damage signal and help coordinating repair with checkpoint activation and cell cycle arrest (Sancar et al., 2004). When the sister chromatid is available as template, repair is addressed through the error-free pathway of homologous recombination (HR) rather than the predominant but error-prone pathway of non-homologous end-joining (NHEJ) (Sancar et al., 2004). HR initiates upon recognition of DNA ends by the MRN complex, an event that facilitates recruitment of ATM through direct interaction with the C-terminus of the NBS1 component (Falck et al., 2005) (Fig. 2A). Recruitment of ATM to chromatin was also reported to occur in response to hypotonic stress-induced chromatin modifications. In this case, however, no physical breaking of the double helix occurs and ATM is recruited by a non-canonical mechanism involving the protein ATMIN (Zhang et al., 2012).

ATM is an homodimer and exists in a complex containing the protein phosphatase PP2A, which maintains ATM inactive by catalyzing its constitutive dephosphorylation (Goodarzi et al., 2004), and the histone acetyltransferase Tip60, which is maintained at low level by CUL3-dependent ubiquitylation and plays a role in the modification of chromatin at sites of damage (Murr et al., 2006;Sun et al., 2009). NBS1-dependent ATM recruitment at sites of damage is followed by ATM autophosphorylation at S_{1981} with ensuing activation of the kinase.

The mechanism of DSB repair operating in the absence of a homologous template for recombination-mediated repair is non-homologous end joining. In this case, DNA ends are bound by the Ku70/Ku80 heterodimer that recruits DNA-PK catalytic subunit, causing inward translocation of the heterodimer and positioning the catalytic subunit at DNA ends. Next, depending on the

complexity of the lesion, different processing factors are recruited, such as the endonuclease Artemis and the polynucleotide kinase/phosphatase PNKP. Autophosphorylation-induced DNA-PKcs release leads to the final step of the process, with XRCC4, DNA ligase IV and XLF performing ligation of the DNA ends (Dobbs et al., 2010).

Site marking - ATM-mediated phosphorylation of H2AX at the C-terminal S_{139} (Rogakou et al., 1998), possibly paralleled by dephosphorylation of Y_{142} (Cook et al., 2009), marks the site of damage and contributes to destabilize nucleosome structure (Fig. 2A). In turn, phosphorylated H2AX acts as docking site for MDC1 that, by virtue of the high affinity of its C-terminal BRCT tandem repeats for the phospho-S_{139} epitope in γH2AX, is the first proteins localizing at sites of damage (Bekker-Jensen and Mailand, 2010). MDC1 orchestrates the consecutive assembly of factors that will, in turn, mediate the recruitment of DNA repair proteins. Such factors comprise 53BP1, BRCA1 and the E3-ubiquitin ligase RNF8. The latter binds phosphorylated MDC1 via its N-terminal FHA domain. SUMO1 modification of HERC2 and RNF168 by the E3-ligase PIAS4 promotes recruitment of RNF8 to the complex and stabilizes the interaction between RNF8 and the E2-conjugating enzyme Ubc13 (Danielsen et al., 2012). In turn, RNF8 contributes to remodel chromatin around sites of damage through a transient K_{48} and a persistent K_{63} ubiquitylation of both H2A and H2AX, facilitating the subsequent recruitment of the DNA repair factors BRCA1 and 53BP1 (Huen et al., 2007;Mailand et al., 2007). Once bound to DNA, MDC1 is sumoylated at K_{1840} by PIAS4 in a manner that facilitates its recognition and ubiquitylation by the E3-ligase RNF4, with consequent degradation (Luo et al., 2012). Additional factors recruited to phosphorylated H2AX consist of chromatin remodeling complexes such as INO80 and SWR1 in yeast (Morrison et al., 2004;van Attikum et al., 2007) and p400 in humans (Xu et al., 2010). K_{63} histone di-ubiquitylation by RNF8, in turn, allows binding of the adaptor protein RAP80 through its UIM motifs (Sato et al., 2009) and the recruitment of Abraxas (ABRA1), which acts as anchor for BRCA1 at sites of DNA damage (Bekker-Jensen and Mailand, 2010). The BRCA1 complex, in turn, contains the DUB BRCC36, which is

able to depolymerize K_{63} ubiquitin chains, thus contributing to maintain steady-state levels of ubiquitin at sites of damage (Shao et al., 2009). The initial histone ubiquitylation by RNF8 represents a docking signal for another E3-ubiquitin ligase, RNF168, which recognizes di-ubiquitylated K_{63} on histone H2A via its MIU domain and amplifies the signal by further ubiquitylating histones around the site of damage (Doil et al., 2009;Pinato et al., 2009;Stewart et al., 2009). The importance of RNF8-/RNF168-dependent ubiquitylation is well exemplified by the RIDDLE syndrome, where recessive mutations in the *RNF168* gene lead to the expression of aberrant RNF168 protein isoforms, resulting in failure of 53BP1 and BRCA1 accumulation at IR-induced foci and of the subsequent activation of DNA damage responses (Stewart et al., 2009). Sumoylation of RNF8, RNF168 and BRCA1 mediated by PIAS1 and PIAS4 enhances their E3-ligase activity, contributing to render more efficient histone ubiquitylation at DSBs (Galanty et al., 2009). Through interaction with the ubiquitin-conjugating UBE2L6/UBCH8, RNF8 controls the degradation of the demethylase JMJD2A/KDM4A resulting in the uncovering of $H4K_{20}$me2 mark and promoting the recruitment of 53BP1 at DNA damage sites (Mallette et al., 2012). RAD18 is another ubiquitin E3-ligase recruited at DNA lesions through recognition of K_{63} ubiquitylated histones and acting downstream of RNF8/ RNF168 (Huang et al., 2009). In concomitance with the events described above, the constitutive phosphorylation of MDC1 by casein kinase 2 (CK2), allows the former to capture additional molecules of ATM that phosphorylate both H2AX in the neighbouring nucleosomes and MDC1 itself (Polo and Jackson, 2011).

Phosphorylation does not only serve the function of facilitating the assembly of modules that become visible in DNA damage-induced foci, but also contributes to break up interactions during repair processes. This is the case of the transcriptional repressor and ring finger protein KAP1/TIF1β/TRIM28, which is released from chromatin upon ATM-mediated phosphorylation of S_{824}, an event that results in the dissociation of heterochromatin protein 1 (HP1) from chromatin and contributes to remodel regions undergoing repair (Goodarzi et al., 2008).

DNA end resection - Marking DNA double-strand break sites is followed by the recruitment of repair proteins in charge of processing DNA ends to create structures that are suitable to recombination. This task is initially accomplished by the MRN complex that in conjunction with CtIP/RBBP8 carries out initial trimming at the break, a step that is followed by extensive processing of DNA ends by the redundant function of EXO1 and the DNA2/BLM complex (Mimitou and Symington, 2009;Eid et al., 2010). Proteins participating in DNA processing are also controlled by PTMs.

CtIP is phosphorylated in a CDK-dependent manner in S and G2 phases of the cell cycle at T_{847} and S_{327}. Whereas phosphorylation of the former affects resection activity, modification of the latter influences BRCA1 binding (Yu and Chen, 2004;Huertas and Jackson, 2009). In response to DSBs CtIP is additionally phosphorylated by ATM (Matsuoka et al., 2007). Binding of BRCA1/BARD1 to CtIP is mediated by the BRCT domain of BRCA1 and causes ubiquitylation of CtIP in a manner that does not target it to degradation but facilitates binding to DNA and enrichment at sites of damage (Yu et al., 2006). This is an example of ubiquitylation as means to selectively target protein to a defined region in the cell or to a structure.

EXO1 protein level is controlled by ATR-dependent phosphorylation and poly-ubiquitylation by an as yet unknown E3 ligase in response to stalled replication (El-Shemerly et al., 2005;El-Shemerly et al., 2008), whereas its exonuclease activity is controlled in a PIKK-dependent manner upon induction of DSBs both in yeast and man (Morin et al., 2008;Bolderson et al., 2010). Sumoylation of EXO1 has also been reported (Tatham et al., 2011), though its functional significance awaits clarification.

The Bloom syndrome helicase (BLM) plays an important role in homologous recombination and in the repair of damaged replication forks (Jones and Petermann, 2012). Modification of BLM by SUMO is necessary for a balanced γH2AX response in HU treated cells, with cells that express SUMO-deficient forms of BLM displaying excessive γH2AX phosphorylation, accumulation of DNA breaks and hypersensitivity to DNA damage (Ouyang et al., 2009). In HU-treated cells expressing SUMO-deficient forms of BLM, the ability to localize RAD51 at damaged replication forks is compromised and sister-

chromatid exchanges does not occur. This led to the suggestion that sumoylation represents a switch between pro- and anti-recombinogenic roles for BLM in HR (Ouyang et al., 2009).

Resection of DNA ends by EXO1 or the BLM/DNA2 complex leads to the formation of long 3'-overhangs that are the structures participating in homologous recombination. Replication Protein A (RPA) is the major ssDNA binding protein complex present in eukaryotes and consists of three subunits: RPA1 (70 kDa), RPA2 (32 kDa) and RPA3 (14 kDa). RPA1 has high affinity for DNA and is the docking subunit for a number of proteins involved in DNA synthesis and repair (Fanning et al., 2006). RPA2 has lower affinity for DNA and, thanks to its C-terminal winged helix domain, binds weakly but specifically to AID, to BER proteins such as UDG or to NER proteins such as XPA (Fanning et al., 2006). RPA2 is the major target of phosphorylation events that occur during DNA replication and the DNA damage response. RPA3 is the only component with no affinity for DNA but playing an important role in the stabilization of the trimeric protein complex (Fanning et al., 2006). The association between the SUMO protease SENP6 and RPA1 during transition through S-phase maintains RPA1 in a hypo-sumoylated state. Camptothecin-induced DSBs weaken the interaction between RPA1 and SENP6, facilitating RPA1 sumoylation at K_{449} and K_{577}, an event that results in increased interaction with Rad51 and displacement of RPA from the ssDNA filament (Dou et al., 2010).

Sumoylation of MRE11 and RAD54 has also been reported (Tatham et al., 2011), though the functional significance of this PTM is as yet unknown.

Proximal and distal signaling - Three members of the PIKK family of protein kinases, namely ATM, ATR and DNA-PK, orchestrate the response to genotoxic damage, consisting in accrual of DNA processing and repair proteins at sites of damage and in cell cycle arrest. Unlike ATM or DNA-PK that are activated by DNA ends (Uematsu et al., 2007;You et al., 2007), ATR activation specifically depends on the presence of ssDNA generated by processing different types of damage (Zou and Elledge, 2003) or naturally occurring at replication forks (MacDougall et al., 2007). The triggering of ATR typically occurs after ATM activation (Jazayeri et al., 2006). The checkpoint

kinases CHK1 and CHK2 are phosphorylated by ATR at S_{317} and S_{345} (Zhao and Piwnica-Worms, 2001) and by ATM or ATR at seven residues in the N-terminal domain (Matsuoka et al., 2000), respectively. This triggers homo-dimerization of the checkpoint kinases and full activation through auto-phosphorylation (Lee and Chung, 2001) (Fig. 2B).

The ATR-CHK1 pathway controls the timing of replication origin firing in S-phase in the absence of DNA damage (Shechter et al., 2004) and triggers G2/M arrest in response to γ-irradiation (Liu et al., 2000). ATR-mediated phosphorylation of CHK1 at S_{345} exposes a degron-like region at the C-terminus of the kinase allowing recognition by the cytoplasmic Cul1/FBX6 or the nuclear Cul4A/CDT2 SCF E3-ligase complexes that promote poly-ubiquitylation and degradation of CHK1 (Zhang et al., 2009;Huh and Piwnica-Worms, 2013). It has been proposed that proteolysis of activated CHK1 results in checkpoint termination (Zhang et al., 2005b).

The ATM/CHK2 axis controls both the transient and the sustained cell cycle arrest that follows detection of DNA damage (Fig. 2B) (Shiloh and Ziv, 2013). Namely, by phosphorylating CDC25 phosphatases and the WEE1 kinase, CHK2 blocks cell cycle transitions mediated by Cyclin-CDKs, whereas by phosphorylating p53, MDM2 and PML it promotes apoptosis (Antoni et al., 2007).

Both CHK1 and CHK2 impinge on the machinery driving cell cycle transitions by directly phosphorylating controllers of cyclin-dependent kinases such as the WEE1 kinase and the CDC25A and CDC25C phosphatases (Bartek et al., 2004) (Fig. 2B). WEE1 catalyzes phosphorylation of two residues in the Gly-rich P-loop of CDK1, namely T_{14} and Y_{15}, in a manner that does not affect nucleotide binding but hampers catalysis (Ferrari, 2006). CDC25 phosphatases specifically remove the phosphate from the two residues in the ATP-binding site of CDKs, causing full activation of Cyclin/CDK complexes (Ferrari, 2006). Inhibition of CDC25C by DNA damage essentially occurs by a 14-3-3-mediated sequestration mechanism, whereas CDC25A degradation via ubiquitin-proteasome pathways is a primary control mechanism both in dividing cells and in response to DNA damage (Donzelli and Draetta, 2003). Phosphorylation of CDC25A on S_{76} by CHK1 (Jin et al., 2008a) serves as

priming event to facilitate phosphorylation on S_{79} and S_{82} by protein kinase CK1 or glycogen synthase kinase-3β (GSK-3β) (Kang et al., 2008;Honaker and Piwnica-Worms, 2010). This, in turn, allows recruitment of the $SCF^{\beta\text{-}TRCP}$ E3 ligase that promotes CDC25A poly-ubiquitylation (Busino et al., 2003).

DNA damage recovery – Following completion of DNA repair, cell cycle restart is contributed by degradation of molecules that were involved both in signaling DNA damage and in blocking cell cycle progression. This is the case of the adaptor protein Claspin, which is targeted by $SCF\text{-}\beta^{TrcP}$ upon PLK1-dependent phosphorylation (Mamely et al., 2006;Peschiaroli et al., 2006) and whose level is maintained low throughout G1 by the APC/CDH1 E3-ligase (Bassermann et al., 2008), and of the kinase WEE1 (Bartek and Lukas, 2007).

Other DNA lesions - Additional examples of regulation of DNA damage responses by ubiquitylation are provided by Fanconi Anemia (FA), Translesion DNA Synthesis (TLS) and Nucleotide Excision Repair (NER). FA is an X-linked disease characterized by mutations in genes coding for factors of this DNA repair pathway. Upon exposure to DNA interstrand cross-linking (ICL) agents, FANC proteins form a nuclear “core-complex” in which FANCL is the E3 ubiquitin ligase responsible, together with its cognate E2 UBE2T, for the mono-ubiquitylation of FANCI and FANCD2 proteins (the ID-complex) on residues K_{561} and K_{523}, respectively, an event required for the formation of damage-induced foci (Wang, 2007). Mutation of the ubiquitin-binding domain on FANCI-FANCD2 results in hypersensitivity to mitomycin C or cisplatin (Smogorzewska et al., 2007). In TLS, which represents one of the main mechanisms allowing DNA lesion bypass in S-phase (Waters et al., 2009), ubiquitylation of PCNA plays a key role (see below). BER addresses the repair of modified bases or abasic sites resulting from depurination/depyrimidinatuon events (Almeida and Sobol, 2007). In addition to TDG (see above), which participates in lesion recognition and processing, the BER scaffold component XRCC1 is controlled by phosphorylation (Loizou et al., 2004) and sumoylation (Gocke et al., 2005). The E3-ligase CHIP/STUB1 adds another layer of control to BER by mediating ubiquitylation

of the pool of XRCC1 and Polβ that are not directly participating in the process of lesion recognition and repair (Parsons et al., 2008).

Interdependence of PTMs

Hierarchical priming - An interesting feature of PTMs is their reciprocal influence, as clearly established for histones, where the antagonism or the synergism of certain modifications defines a "code" that guides protein-DNA interactions (Sims and Reinberg, 2008). These, in turn, influence the compaction of chromatin and ultimately affect biological responses such as transcription, DNA replication and DNA repair (Kouzarides, 2007). Such effects can be cumulative or exclusive, with a clearly defined hierarchy of PTMs affecting a given target protein. In the DDR, a notable example of consecutive PTMs occurring in a hierarchical manner is represented by FEN1, the flap endonuclease responsible for cleavage of single stranded 5' overhangs in Okazaki fragments during DNA replication and also involved in DNA repair. Phosphorylation at S_{187} in FEN1 catalytic domain by cyclin A/CDK2 results in its release from PCNA, the DNA polymerase processivity factor that stimulates FEN1 nuclease activity (Henneke et al., 2003). Subsequent modification of K_{168} in FEN1 by SUMO3 facilitates K_{354} ubiquitylation by the E3 ligase PRP19, resulting in FEN1 degradation at the end of S-phase, an event that contributes to ensure a timely transition to G2 (Guo et al., 2012).

Competition for the substrate - In addition to the ability of sumoylation to directly alter the properties of the protein undergoing this modification, it may also serve as a competitor to other PTMs. Indeed, since sumoylation targets lysine residues in the substrate, similarly to ubiquitylation, methylation or acetylation, the modification of one or more lysine in the substrate could block access to other PTM machineries, thus indirectly affecting protein function (Walsh et al., 2005). Established examples of competition among PTMs are RanGAP1, which upon sumoylation preferentially binds the nuclear pore

complex (Melchior, 2000), the NF-κB signaling pathway (Huang et al., 2003) and PCNA (Hoege et al., 2002). Specifically to the latter, early studies showed that mono-ubiquitylation mediated by RAD6 (E2) and RAD18 (E3), K_{63} poly-ubiquitylation by MMS2, UBC13 and RAD5 and sumoylation by UBC9 all affect the same lysine residue (K_{164}) (Hoege et al., 2002). Subsequent work clearly established that PCNA mono-ubiquitylation supports translesion synthesis, a pathway allowing stalled DNA replication to proceed beyond damage through the replacement of processive polymerases with specialized polymerases (Bienko et al., 2005;Garg and Burgers, 2005). K_{63} poly-ubiquitylation, on the other hand, facilitates synthesis by a template-switch mechanism, a complex but essentially error-free pathway that utilizes the undamaged, newly synthesized daughter strand of the sister chromosome as template (Branzei and Foiani, 2010). Finally, PCNA SUMOylation prevents the formation of DSBs and the occurrence of inappropriate recombination events at stalled DNA replication forks by a mechanism involving the anti-ricombinogenic activity of the helicase Srs2 in yeast (Papouli et al., 2005;Pfander et al., 2005) and possibly by a similar mechanism in humans (Gali et al., 2012).

Cross-talking - A number of ubiquitin E3-ligases display the ability to bind SUMO chains on proteins that, in turn, become their substrates. A reported case is PML, which undergoes modification by SUMO-1 as well as by SUMO-2/3. Whereas attachment of SUMO-1 determines confinement of the protein in PML nuclear bodies (Muller et al., 1998) formation of SUMO2/3 chains facilitates the recruitment of the E3-ligase RNF4, which ubiquitylates the SUMO chains ultimately targeting PML to degradation (Lallemand-Breitenbach et al., 2008;Tatham et al., 2008;Weisshaar et al., 2008). RNF4 displays the ability to interact with other sumoylated substrates, such as MDC1 and RPA, via its N-terminal SUMO interaction motif (SIM) and to subsequently regulate their stability (Galanty et al., 2012). Another interesting case is BRCA1, which co-localizes with and is sumoylated by PIAS1 and PIAS4 at sites of damage. This, in turn, was reported to enhance BRCA1 E3-ligase activity possibly through a SUMO-dependent increase of the E3-E2

interface (Morris et al., 2009)

Synergy - The advent of proteome-wide studies allowed appreciating the fact that, like phosphorylation, sumoylation triggered by a defined stimulus or stress does not target a single components but a vast majority of the protein machinery involved in the response. Work conducted in yeast established that lack of overall sumoylation in a hypomorphic mutant of the SUMO E2 Ubc9 impaired survival in response to DNA damage (Cremona et al., 2012). This resulted from incomplete replication of damaged DNA as well as defective resection at DSBs. The authors found that DNA damage-induced sumoylation occurred independently of phosphorylation events that were triggered by the checkpoint and was proposed to act in parallel with them to support cell survival (Cremona et al., 2012). A study conducted using SILAC-based mass spectrometry identified 844 different SUMO conjugates, the abundance of which did not seem to change in response to DNA damage (Psakhye and Jentsch, 2012). Interestingly though, the set of sumoylated proteins enriched in response to DNA damage was specifically that of the HR machinery. The authors found that DNA end resection and the consecutive generation of long ssDNA tracts acted as trigger to the wave of sumoylation that characterized the response. Sumoylation of HR proteins was found to occur independently and in parallel, with no influence of one sumoylation event on the other, and to entirely depend on the SUMO E3-ligase Siz2. Sumoylation promoted physical interaction among HR proteins, thus facilitating DNA repair (Psakhye and Jentsch, 2012).

Ubiquitin and SUMO as targets in cancer therapy

Ubiquitylation and/or sumoylation defects have been implicated in the pathogenesis of a number of human diseases among which is cancer (Sun, 2006;Bettermann et al., 2012).

Ubiquitin and cancer - Examples of over-expression of ubiquitinylation pathway components in cancer cells are the p53-specific ARF-BP1/Mule HECT E3-ligase, the F-box proteins SKP2 and β-TrcP1, the SCF component

Cul-4A and the RING-finger proteins RNF11, ZNF164 (Chen et al., 2006) and RNF5 (Bromberg et al., 2007). Mutation or deletion of E3-ligases that normally function as tumor suppressors has also been reported. This is the case of the RING-finger E3-ligases BRCA1/BARD1 and SIAH1 (Chen et al., 2006). Finally, epigenetic inactivation of genes coding for the E3-ligase HACE1 (Hibi et al., 2008) or the RING-finger protein CHFR (Chen et al., 2006) has been observed in several types of carcinomas.

Based on the reasoning that E3 enzymes are druggable targets, pharmaceutical companies embarked on high-throughput screenings in search for compounds that would target the active site of E3-ligases or block interaction with their substrates (Sun, 2006;Hoeller and Dikic, 2009). A notable example of the latter is Nutlin, which impairs the p53-HDM2 interaction by filling a groove in HDM2 where p53 is accommodated (Vassilev, 2007). Despite the initial enthusiasm raised by Nutlin and its derivatives, the limitation of its efficacy in cells expressing wild-type p53 excluded their use in a number of other cancers. More discouraging, the cytostatic effect of Nutlins in p53-deficient cells indicated that they did not solely inhibit the p53/HDM2 interaction (VanderBorght et al., 2006). The p53-targeting molecule RITA (NSC652287), identified in a screening conducted on a pair of isogenic cell lines differing only in their p53 status, was shown to bind p53 N-terminus (Issaeva et al., 2004). However, RITA did not specifically target the p53-HDM2 dimer but also other p53 protein complexes (Hjerpe and Rodriguez, 2008). Similar issues were encountered with other inhibitors of E3-ligases (Guedat and Colland, 2007).

The E1-activating enzyme and the proteasome have also been considered as possible targets, with the *caveat* that inhibiting the ubiquitylation cascade at its apex may impair pathways of vital importance to the survival of normal cells. This is particularly true if one considers the widespread use of ubiquitylation in the control of cellular functions. Nonetheless, inhibitors of the chymotryptic activity of the proteasome have been identified and characterized. Compounds such as bortezomib have received approval from FDA and are currently used for the treatment of multiple myeloma and mantle cell lymphoma (Guedat and Colland, 2007;Rastogi and Mishra, 2012). Similarly, ATP-competitive inhibitors blocking the transfer of ubiquitin from the E1-

activating enzyme to E2-conjugating components of the cascade have been identified (Guedat and Colland, 2007). Inhibition of deubiquitylating enzymes has also been explored as possible alternative to the development of inhibitors of the ubiquitylation cascade. A compound specifically targeting USP7 was shown to stabilize p53, activate p53-dependent transcription, block cell growth and induce apopotosis (Guedat and Colland, 2007). Recently, a novel strategy based on the use of combinatorial libraries of ubiquitin variants has led to the identification of mechanisms of DUBs inhibition and provided the demonstration that this approach could be applied to the discovery of specific E2 or E3 inhibitors (Ernst et al., 2013).

SUMO and cancer - With regard to the role of SUMO in cancer, Ubc9/UBE2I was found overexpressed in ovarian carcinoma specimens (Mo et al., 2005). Xenografts studies conducted in mice revealed that tumors expressing wildtype Ubc9 grew better than controls, while tumors expressing dominant negative Ubc9 exhibited reduced growth (Mo et al., 2005). A comprehensive study reported an increase in UBC9 expression in primary colon and prostate cancer compared with their normal tissue counterparts, whereas UBC9 levels were found lower in metastatic breast, prostate, and lung cancer in comparison with their corresponding normal and primary adenocarcinoma tissues (Moschos et al., 2010). Increased UBC9 expression was also observed in melanoma-infiltrated lymph nodes, with depletion of UBC9 resulting in sensitization of melanomas to the cytotoxic effects of topotecan and cisplatin (Moschos et al., 2007). A comprehensive collection of studies on UBC9 mRNA expression pattern in different cancer types can be found at www.nextbio.com. Based on these findings, targeting UBC9 in cancer therapy was initially proposed (Mo et al., 2005). However, given the widespread use of sumoylation as PTM controlling numerous metabolic pathways, altering the overall pattern of sumoylation in the cell was countered by others as a non-specific and ineffective method to combat cancer (Bawa-Khalfe and Yeh, 2010). In support of arguments to UBC9 as valid target in cancer therapy is the differential expression issue, with higher levels of UBC9 in cancerous vs. normal tissues offering a possible therapeutic window (Mo and Moschos, 2005). In this respect, crystallographic studies mapping the surfaces in UBC9

involved in the interaction with specific E3s and their substrates represent a promising avenue to the design of small compounds disrupting selective sumoylation reactions (Mo and Moschos, 2005).

Increased levels of the desumoylating enzyme SENP1 were reported in thyroid oncocytic adenocarcinoma (Jacques et al., 2005) and prostate cancer (Cheng et al., 2006). A transgenic mice model showed that overexpression of Senp1 in the prostate led to the development of prostatic intraepithelial neoplasia at an early age (Cheng et al., 2006). Promising results have been obtained in studies aiming at the identification of SUMO-specific protease (SENP) inhibitors (Hemelaar et al., 2004;Borodovsky et al., 2005) or based on the screening of cysteine-protease inhibitor libraries (Albrow et al., 2011). The latter, in particular, led to the identification of two classes of compounds: the first, containing a reactive aza-epoxide electrophile linked to an extended peptide backbone and the second, containing an acyloxymethyl ketone reactive group. Structure-activity relationship studies led to the design of covalent inhibitors of multiple hSENPs displaying micromolar IC_{50} values (Albrow et al., 2011).

Arsenic trioxide, which induces differentiation of leukemic blasts and clinical remission, was shown to promote SUMO-dependent poly-ubiquitylation of PML-RARα by the ubiquitin E3-ligase RNF4, with consequent degradation of the fusion protein responsible for acute promyelocytic leukemia (Lallemand-Breitenbach et al., 2008;Tatham et al., 2008). Thus, in addition to classic approaches based on the chemical inhibition of enzymatic activity, the case of arsenic trioxide illustrated that among the variety of possible avenues to inhibit function, the exploitation of existing pathways in the cell that may be triggered at will, is an important option.

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FIGURE LEGENDS

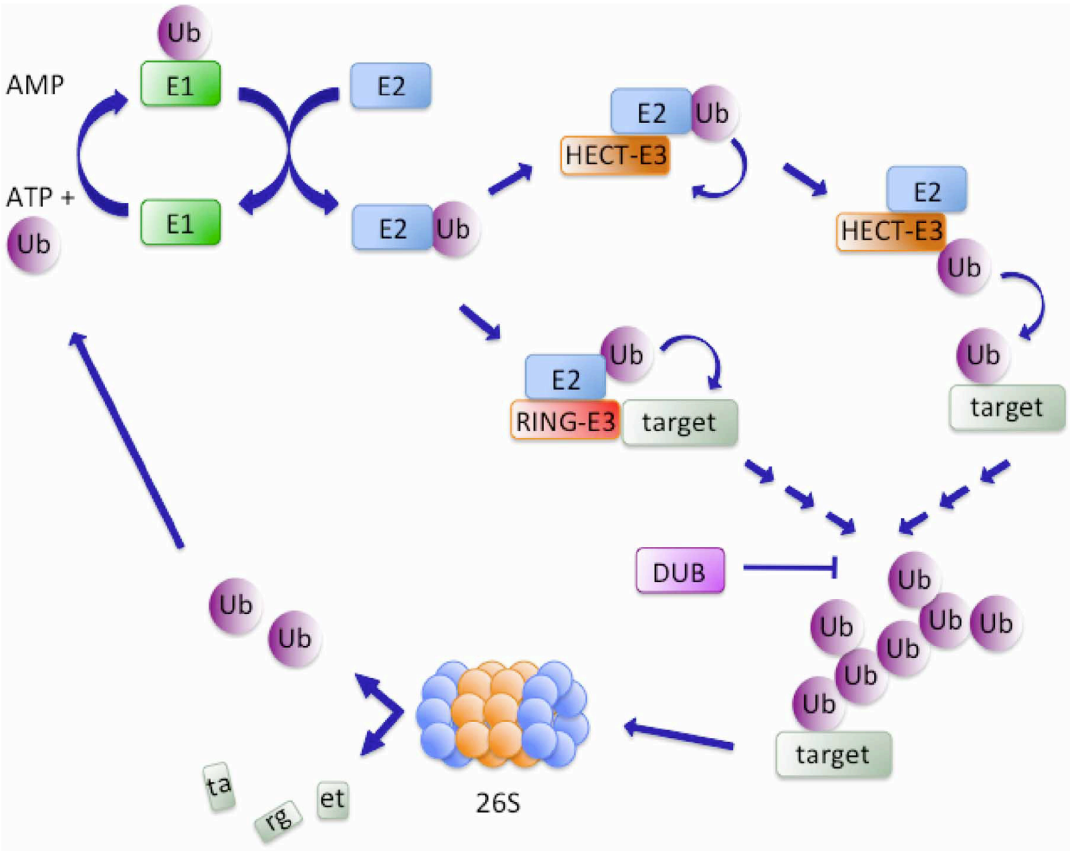
Figure 1 – Simplified scheme of ubiquitylation

The ubiquitylation cascade initiates with an ATP-dependent reaction consisting in the formation of a thiolester bond between a cysteine in the active site of the E1-activating enzyme and G_{76} in ubiquitin (Ub). In the second step, ubiquitin is transferred to the active cysteine of an E2-conjugating enzyme. Finally, an E3-ligase enzyme binds the E2-Ub complex and transfers ubiquitin to lysine residues of the acceptor substrate (target), which is shuttled to the 26S proteasome. Ubiquitin is recycled for another round of reactions.

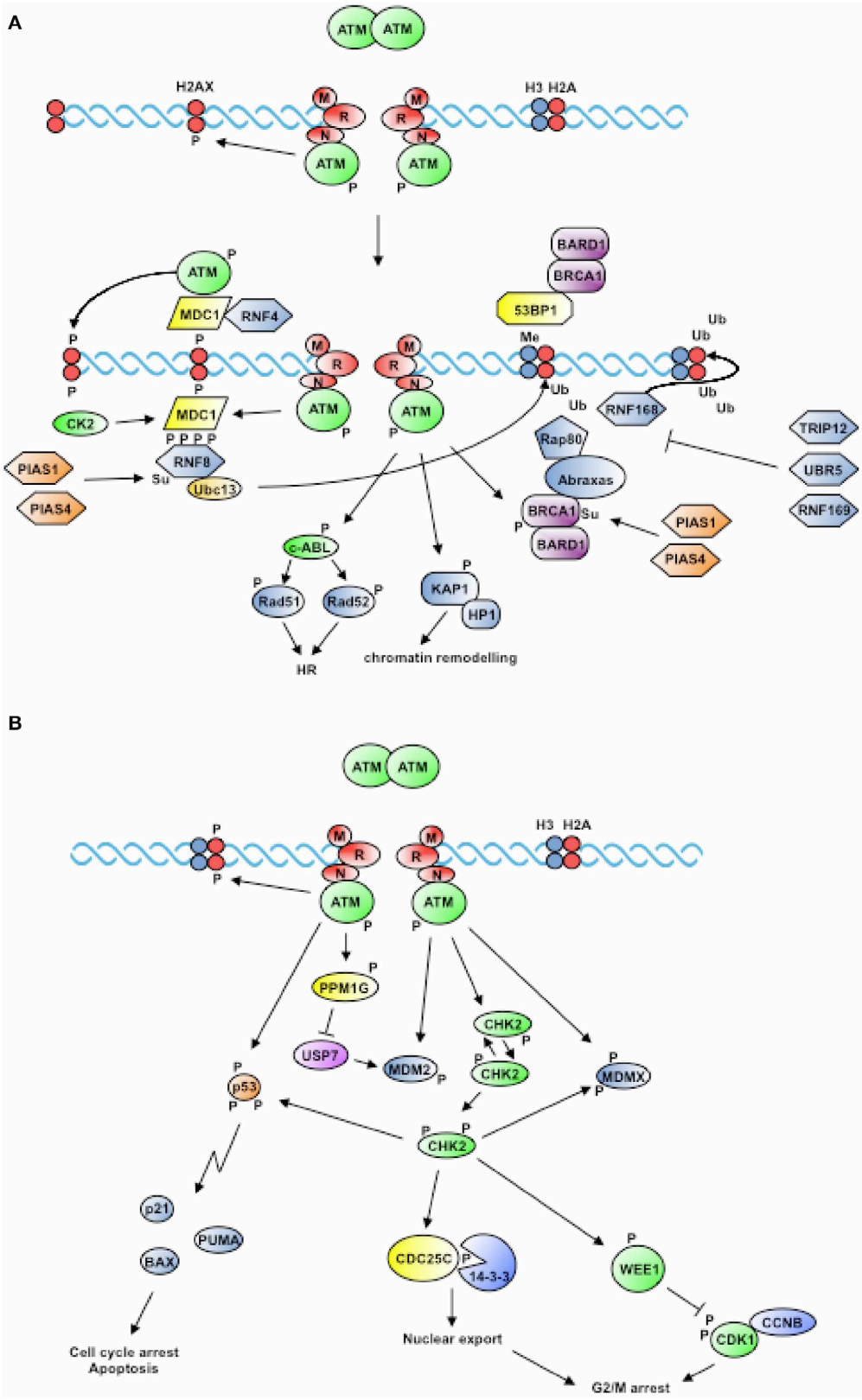
Figure 2 – Proximal and widespread DNA damage signals

(A) In response to the generation of DSBs, ATM is recruited to DNA in an MRN-dependent manner and is activated by autophosphorylation. ATM-dependent phosphorylation of H2AX triggers the recruitment of factors that mark the site of damage and cooperate to amplify the signal. In addition, ATM phosphorylates proteins that contribute to remodel chromatin and promote homologous recombination (see text for details).

(B) Activation of ATM triggers the phosphorylation of the protein kinase CHK2 among others, which freely diffuses from the site of damage to transduce DNA damage signals to cell cycle regulators, resulting in the inhibition of cell cycle transitions (see text for details).



Bologna et al., Figure 1



Bologna et al., Figure 2

3. AIM OF STUDY

The genetic information contained in DNA is duplicated and faithfully segregated into daughter cells during a cycle of cell division. Tight control of factors involved in recognition and repair of DNA damage is of key importance to maintain a stable genome. Exonuclease-1 (EXO1) is one such factor. Exonuclease 1 (EXO1) is an exonucleolytic enzyme and a common component of machineries processing stalled replication forks, DSBs and DNA base mismatches. Indeed, it resects DNA at site of breaks from 5' to 3' producing 3' overhanging ssDNA filaments that are substrates for the components of the DNA repair machinery by homologous recombination.
Previous work from our laboratory demonstrated that the function of human and yeast EXO1 at DSBs and stalled forks, respectively, is rigorously controlled by specific protein-protein interactions (Eid et al., 2010; Engels et al., 2011). Additional studies from our laboratory showed that, in response to stalled DNA replication, the cellular level of human EXO1 is regulated by phosphorylation-dependent ubiquitylation that channels EXO1 to proteasome-mediated degradation (El-Shemerly et al., 2008; El-Shemerly et al., 2005).

The aim of my project is to extends these findings and, taking advantage of a combination of molecular biology and biochemical techniques as well as cell biology assays, to provide new mechanistic insights on the regulation of EXO1 by identification of the main players involved in EXO1 post-translational modification upon DNA damage. Furthermore my final goal is to elucidate the effects of EXO1 stabilization and genome stability.

Elucidating the mechanism regulating EXO1 upon double-strand breaks or stalling of the replication forks.

4.1 Sumoylation regulates EXO1-dependent resection at sites of DNA damage

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Sumoylation regulates EXO1-dependent resection at sites of DNA damage

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ABSTRACT

Processing DNA double-strand breaks (DSBs) by the error-free pathway of homologous recombination (HR) requires the concerted action of a number of factors. Among these, EXO1 and DNA2/BLM are responsible for the extensive resection of DNA ends to produce 3'-overhangs, which are essential intermediates for later HR steps. Here we address the role of post-translational modifications (PTMs) in the control of EXO1 at sites of DNA damage. We show that EXO1 is a SUMO target and that sumoylation affects EXO1 protein stability under basal as well as DNA damage conditions. We identify an UBC9-PIAS1/PIAS4-dependent mechanism controlling human EXO1 sumoylation *in vivo* and, using an *in vitro* reconstituted system, demonstrate conservation of this mechanism in yeast by the Ubc9-Siz1/Siz2. Furthermore, we show physical interaction between the desumoylating enzyme SENP6 and EXO1, promoting EXO1 stability. We provide evidence that sumoylation and ubiquitylation occur sequentially on EXO1 in response to DNA damage and that the former affects recruitment of EXO1 to sites of damage. Finally, we identify the major sites of sumoylation in EXO1 and show that ectopic expression of a sumoylation-deficient form of EXO1 rescues the DNA damage-induced chromosomal aberrations observed upon wt-EXO1 expression. Thus, our study identifies a novel layer of regulation of this DNA damage response protein by elucidating the cross-talk between sumoylation and ubiquitylation, making EXO1 and the pathways regulating its function an ideal target for therapeutic intervention.

INTRODUCTION

Faithful repair of DNA lesions is essential to the maintenance of genome stability [1]. Double-strand breaks (DSBs) are the most toxic DNA lesions generated by ionizing radiation (IR), certain chemotherapeutic drugs, collapse of stalled DNA replication forks, endogenous metabolic processes or during meiotic recombination [2-4]. Inappropriate repair of DSBs may cause gross chromosomal aberrations [5] resulting in carcinogenesis through activation of oncogenes or inactivation of tumor suppressor genes [6]. Cells utilize two main mechanisms to repair DSBs: non-homologous end-joining (NHEJ) and homologous recombination (HR) [7]. Rejoining of DSBs by NHEJ takes place throughout the cell cycle, whereas HR is restricted to the S and G2 phases, where sister chromatids are available as templates. HR is initiated by 5'-3' resection of DSBs to produce single-stranded DNA (ssDNA) tails that function not only as a signal for the ATR-mediated DNA damage checkpoint but also to allow formation of RAD51 filaments and recruitment of recombination proteins [8]. Studies conducted in yeast and mammalian cells led to the proposal of a mechanism according to which MRN and CtIP (MRX and Sae2 in yeast) orchestrate the initial trimming of DNA-ends, which is followed by a processive step of resection carried out by two alternative pathways that involve either EXO1 or BLM/DNA2 [9].

EXO1 was originally identified in *S. pombe* [10] and subsequently in humans [11]. It belongs to the Rad2 family of DNA repair nucleases and is able to remove mononucleotides from the 5' end of the DNA duplex [12]. EXO1 is implicated in several DNA repair pathways including mismatch repair, post-replication repair, meiotic and mitotic recombination and double-strand break repair [13-17]. *S. cerevisiae* Exo1 acts redundantly with Rad27 in processing Okazaki fragments during DNA replication [18]. More recently, Exo1 was shown to be recruited to stalled replication forks where it counteracts fork reversal [19].

The recruitment of proteins that mark sites of DNA damage as well as the choice of pathways addressing their repair heavily relies on control by PTMs that occur in a defined hierarchy [20]. An archetypal example is the phosphorylation-dependent binding of MDC1 to γH2AX, followed by

recruitment of the E3-ubiquitin ligases RNF8 and RNF168 whose activity is enhanced by PIAS1- and PIAS4-mediated sumoylation, resulting in efficient H2A and H2B mono-ubiquitylation [20]. An interesting feature of PTMs is not only the hierarchical level at which they occur but also their reciprocal influence, as demonstrated for FEN1 [21].

We have previously shown that the function of human and yeast EXO1 at DSBs and stalled forks is rigorously controlled by interaction with CtIP and 14-3-3 proteins, respectively [5,22]. Additionally, human EXO1 activity is controlled by post-translational modifications, with ATR-dependent phosphorylation targeting it to ubiquitin-mediated degradation upon replication fork stalling [23,24], and ATM-dependent phosphorylation apparently restraining its activity during homologous recombination [25]. Analogously, Mec1-dependent phosphorylation inhibits yeast Exo1 activity at uncapped telomeres [26].

In this study we have focused on elucidating the molecular mechanism of EXO1 regulation upon stalled DNA replication. We have performed an RNAi-based screen of human E2-conjugating enzymes and identified UBE2I, the human homolog of yeast Ubc9, as major effector of EXO1 stability. We show that EXO1 is sumoylated *in vivo* in a PIAS1- and PIAS4-dependent manner and *in vitro* using a reconstituted system. We also provide evidence that SENP6 physically interacts with EXO1 and that depletion of SENP6 promotes EXO1 degradation. Furthermore, identification of the major SUMO-conjugation sites in EXO1 allowed us to demonstrate that sumoylation affects the time of EXO1 residence at sites of DNA damage. Finally, we observed that the high rate of chromosome breaks caused by camptothecin in cells ectopically expressing wild-type EXO1 did not occur upon expression of a SUMO-deficient EXO1 mutant.

RESULTS

EXO1 is degraded in response to camptothecin – In response to agents that cause stalling of DNA replication forks, such as hydroxyurea (HU) or aphidicolin (APH), human EXO1 undergoes ubiquitylation-dependent proteasomal degradation [23,24]. To substantiate and extend the observation that EXO1 degradation occurs upon DNA damage and is S-phase-specific, we additonally verified the effect of the topoisomerase-1 inhibitor camptothecin (CPT). As shown in Fig. 1a, a 4h CPT treatment led to a net decrease of EXO1 protein level and this effect was rescued by the presence of the proteasome inhibitor MG-132. Given the low level of EXO1 expression in mammalian cells, which requires combined immunoprecipitation and Western blot to visualize the protein (Fig. 1a and [23]), we asked whether ectopically expressed EXO1 would behave as the endogenous protein. To this end, we first performed a dose-response study on HEK-293T cells transiently expressing GFP-EXO1. Similarly to the endogenous protein, exogenous GFP-EXO1 was sensitive to CPT-treatment already at the lowest concentration tested (Fig. 1b and 1c). The ability of CPT to cause a decrease in EXO1 protein level was confirmed in U2OS cells stably expressing EXO1 (U2OS-GFP-EXO1)[5] by either Western blot analysis (Fig. 1c) or immunofluorescence (Fig. 1d). Addition of the proteasome inhibitor MG-132 or the E1-Ubiquitin-activating enzyme inhibitor PYR-41 rescued CPT-induced EXO1 degradation (Fig. 1c and 1e).

Taken together, these data indicate that both endogenous and exogenous EXO1 are targets for ubiquitin-dependent proteasomal degradation in response to topoisomerase-I inhibition.

EXO1 degradation depends on SUMO pathways – In order to shed light on the mechanism controlling EXO1 protein stability, we set out to identify the pathway responsible for EXO1 degradation. To this end, we performed an immunofluorescence-based high-throughput screen of an siRNA library of all 37 human E2 ubiquitin-conjugating enzymes in U2OS-GFP-EXO1 cells, followed by image and computational analysis of the acquired data. The most significant increase in green fluorescence, taken as read-out for GFP-EXO1

protein stabilization, was obtained upon depletion of UBE2I (the human homologue of the yeast Ubc9 enzyme, referred to as UBC9), both in basal and damaging conditions (Fig. 2a). We could exclude any indirect effect of UBC9 depletion on cell size and cell cycle phases (Fig. S1). To corroborate the role of UBC9, we examined EXO1 stability in U2OS cells depleted for UBC9 with a single siRNA. Under these conditions, Western blotting (Fig. 2b) and indirect immunofluorescence (Fig. 2d) confirmed an increase in GFP-EXO1 protein level in both untreated and CPT-treated cells. It should be pointed out that stabilization of GFP-EXO1 in non-damaged cells that were depleted for UBC9 is consistent with the fact that EXO1 undergoes constitutive degradation, as we previously demonstrated by chemical inhibition of the proteasome with MG-132 or in ATR-deficient Seckle cells [23,24] Quantification of GFP-positive cells in untreated conditions showed a 3.1-fold increase upon depletion of UBC9 (Fig. 2d and S2a). UBC9-depleted cells showed a 1.4-fold increase in fluorescence signal compared to control-depleted cells (Fig. S2b). Similar values were observed upon CPT treatment (data not shown). An analogous pattern of protein stabilization was confirmed in UBC9-depleted HEK-293T cells ectopically expressing GFP-EXO1 (Fig. 2c). To corroborate these observations, we co-expressed HA-UBC9 and GFP-EXO1 in HEK-293T cells, which resulted in clear reduction of GFP-EXO1 protein level (Fig. 2e). The minimal EXO1 degradation in response to DNA damage observed in this case (Fig. 2e lane 1 *vs.* 2) is likely due to saturation of proteasome-dependent degradation machinery in over-expression studies.
Taken together, these data indicate that EXO1 protein level is controlled by SUMO pathways both in unperturbed conditions and upon stalled DNA replication.

EXO1 is a direct target of SUMO pathways – Next, we examined whether EXO1 is a direct target for sumoylation. To this end, we performed *in vitro* studies with a reconstituted sumoylation machinery. Using purified recombinant components of the yeast machinery, which is a very robust system for such enzymatic assays, we observed that both yeast and human EXO1 are modified by SUMO (Fig. 3a and 3b). Considering the role of EXO1 in DNA repair pathways, we asked whether E3-SUMO ligases with an

established role in the DNA damage response would affect EXO1 sumoylation. We observed that the presence of the E3-SUMO ligases Siz1 or Siz2 was essential for the sumoylation of yeast Exo1 and significantly increased the pattern of human EXO1 sumoylation obtained in the absence of a specific E3 enzyme (Fig. 3a and 3b). *In vitro* assays performed with purified recombinant components of the human SUMO machinery (Fig. 3c) confirmed that human EXO1 is also target of sumoylation (Fig. 3d).

To extend these observations to a cellular system, we ectopically expressed Myc-SUMO1 and Myc-SUMO2 along with GFP-EXO1 in HEK293T cells. Immunoprecipitation of EXO1 followed by probing with a monoclonal antibody to the MYC tag showed that EXO1 is constitutively sumoylated and that sumoylation slightly increases in response to stalled DNA replication (Fig. 4a). To distinguish between effects of SUMO1 and SUMO2 on EXO1, we examined HEK293T cells ectopically expressing either one of the two proteins along with GFP-EXO1. Data indicated that SUMO1, despite being expressed at a significantly lower level than SUMO2 in all experiments that we performed, caused a more pronounced decrease of GFP-EXO1 protein level than did SUMO2 (Fig. 4b). To follow up the observation that Siz1 and Siz2 facilitate EXO1 sumoylation *in vitro* (Fig. 3a and 3b), we depleted PIAS1 or PIAS4, the human E3-SUMO ligases homologue of Siz enzymes, in HEK293T cells. Data showed that downregulation of PIAS1 or PIAS4 rescued GFP-EXO1 protein level in response to CPT, with a clear increase of the protein also in unperturbed conditions (Fig. 4c).

Since SENP proteases possess both endopeptidase activity, required for the maturation of SUMO1-3 precursors, and isopeptidase activity, necessary for reverting protein sumoylation [20], we wished to test their effect on EXO1 stability. RNAi-mediated depletion of SENP proteases in stable U2OS cells (data not shown) revealed that SENP6 downregulation caused a decrease of GFP-EXO1 protein stability (Fig. 4d and S3). Protein interaction studies in HEK-293T cells ectopically expressing Flag-SENP6 and GFP-EXO1 showed that the two proteins could be co-immunoprecipitated, in both untreated and CPT-treated conditions, indicative of a physical interaction (Fig. 4e). Pull-down studies with recombinant proteins showed that intein-tagged EXO1,

which was affinity-purified with chitin beads, was able to capture soluble SENP6 protein (Fig. 4f).
Taken together, these data indicate a functional role for the E3-SUMO ligases PIAS1/PIAS4 and the desumoylating enzyme SENP6 on EXO1 protein stability.

Sumoylation and ubiquitylation of EXO1 occur sequentially - To further investigate hierarchy as well as functional links between sumoylation and ubiquitylation [20], we examined GFP-EXO1 in stable U2OS cells that were transfected with HA-Ubiquitin and treated with hydroxyurea (HU), an inhibitor of DNA replication that we have previously shown to induce EXO1 ubiquitylation [23,24]. Immunoprecipitation of EXO1 revealed that the HU-induced increase in ubiquitylation pattern was significantly diminished upon treatment of the cells with anacardic acid, the E1-SUMO-activating enzyme inhibitor (Fig. 5a). In agreement, the reduction of ubiquitylation observed under these conditions was similar to that obtained upon treatment of the cells with PYR-41, the E1-UBI-activating enzyme inhibitor (Fig. 5a), indicating that sumoylation may precede modification by ubiquitylation. To corroborate these findings, we examined the extent of EXO1 ubiquitylation upon down-regulation of UBC9 expression. As shown in Fig. 5b, the pattern of HU-induced EXO1 ubiquitylation observed in control siRNA-treated cells was clearly reduced upon UBC9 depletion. This effect is particularly remarkable, considering the overall increase in the level of EXO1 protein in UBC9-depleted cells (Fig. 5b).
Taken together, these data establish that sumoylation is a prerequisite for ubiquitin-mediated degradation of EXO1.

Sumoylation affects EXO1 recruitment to chromatin – PTMs affect protein properties such as stability, localization and binding to DNA [20,27]. We and others have previously described the rapid and transient localization of EXO1 to DSBs generated by laser micro-irradiation of nuclei [5,25]. To assess whether sumoylation would influence EXO1 recruitment to DNA, we examined the DNA binding properties of recombinant nuclease-deficient EXO1 mutant, in order to exclude degradation of the substrate, upon *in vitro* sumoylation. Data

showed that the ability to bind a synthetic DNA substrate was not significantly affected by sumoylation (Fig. 6a). Next, we examined the pattern of EXO1 immunostaining in stable U2OS cells damaged by laser micro-irradiation to determine the effect of sumoylation *in vivo*. GFP-EXO1 was able to localize to sites of DNA damage within 5 min of irradiation in both control and UBC9-depleted cells with a signal still detectable 30 min post-irradiation (Fig. 6b and 6c), confirming previously published data [5]. Notably, the entire population of GFP-EXO1 co-localized with γ-H2AX in siCTRL-depleted cells, whereas a significant proportion of GFP-EXO1 molecules remained diffused through the nucleus in siUBC9-depleted cells (Fig. 6b, inset).

To substantiate these observations, we examined the chromatin-bound fraction of GFP-EXO1 in HEK293T cells that were treated with CPT. Data showed that, whereas the total population of GFP-EXO1 underwent almost complete degradation in response to CPT, the chromatin-bound population was comparable between untreated and treated cells (Fig. 6d), indicating that the low number of GFP-EXO1 molecules present upon damage is fully localized to chromatin. This population is regulated by ubiquitylation, as demonstrated by the increase in chromatin-bound EXO1 upon treatment with MG-132 (Fig. 6d). Furthermore, UBC9 depletion caused a great increase of the overall GFP-EXO1 population (Fig. 6e, see also Fig. 2a-d). Whereas in untreated cells a majority of GFP-EXO1 molecules were chromatin-bound, likely due to EXO1 role in other DNA repair pathways than in homologous recombination, no signal for GFP-EXO1 was detected on chromatin upon CPT treatment (Fig. 6e), indicating a specific role for sumoylation in EXO1 recruitment to DSBs.

Hence, while *in vitro* sumoylation does not have a direct effect on DNA binding, *in vivo* experiments have shown that sumoylation facilitates the recruitment of EXO1 to sites of DNA damage, indicating that the complexity of events occurring in the cell cannot be recapitulated in a minimal reconstituted system.

Identification of sumoylation sites in EXO1 - In order to assess the physiological role of sumoylation, we set out to identify the SUMO conjugation

sites in EXO1. Analysis of EXO1 primary sequence revealed the presence of four potential sumoylation sites corresponding to the canonical consensus motif F-K-x-E/D, with two consecutive lysine residues located at the C-terminus of the protein (Fig. 7a). Mass spectrometric analysis of *in vitro* sumoylated EXO1 revealed that lysine K_{655} and K_{801} were modified by SUMO (Fig. S4a and S4b). Hence, we generated the $K_{655}/K_{801/K802}$>R mutant (EXO1-3KR) as well as the $K_{48/185/655/801/K802}$>R mutant (EXO1-5KR) where all predicted sites were mutated. *In vitro* assays conducted on purified recombinant EXO1-3KR as well as EXO1-5KR showed that the sumoylation pattern observed in wt-EXO1 was essentially abolished in the mutants (Fig. 7b and data not shown). While binding of EXO1-3KR to a synthetic DNA substrate was similar to that observed for the wild-type protein (Fig. 7c), the DNA resection activity of EXO1-5KR (Fig. S5) appeared to be lower than that of wt-EXO1 and EXO1-3KR (Fig. 7d). This may reflect mutation of lysine 185 in the catalytic domain of EXO1 (Fig. 7a). For this reason, the EXO1-5KR mutant was not further considered.

In summary, we have identified the SUMO-conjugation sites in EXO1 protein and characterized the corresponding SUMO-deficient mutant.

Effect of sumoylation on DNA resection and genome stability –To evaluate the effect of sumoylation on the above-mentioned EXO1 activities, we first examined DNA resection using a synthetic DNA substrate. Despite the high stoichiometry of EXO1 modification by SUMO, DNA resection activity was apparently not affected (Fig. 8a and 8b).

However, given that the complexity of protein interactions occurring in the cell cannot be reconstituted in a simplified *in vitro* system, we decided to assess the effect of EXO1 sumoylation on DNA processing *in vivo* coupling quantification of DNA resection (phospho-RPA) with analysis of DNA content (DAPI) by flow cytometry. In comparison to mock-transfected cells, U2OS ectopically expressing GFP-EXO1-wt displayed a two-fold increase of DNA-bound RPA under basal conditions (Fig. 8c and 8d). A similar pattern was observed in untreated and HU-treated HeLa cells transiently expressing GFP-EXO1-wt (Fig. S6).

To appreciate the biological significance of increased DNA resection by EXO1, we examined chromosomal abnormalities in cells ectopically expressing GFP-EXO1-wt or the GFP-EXO1-3KR mutant. Transfected cells were treated with CPT to cause DNA damage, caffeine was added to override the G2/M checkpoint and chromosome spreads were obtained upon arresting cells in metaphase with colcemid. CPT-treatment of GFP-EXO1-wt expressing cells resulted in a significant amount of aberrations, mostly consisting of breaks and fragments (Fig. 8e and S7). This pattern was clearly reduced in cells expressing the GFP-EXO1-3KR mutant (Fig. 8e and S7). Hence, from these data we conclude that sumoylation-mediated targeting to DNA is essential for EXO1 to exert its function.

DISCUSSION

Genetic conditions characterized by dysfunction of the machinery that signals DNA damage and/or addresses its repair are associated with a predisposition to the development of cancer and resistance to therapy [6], providing a direct demonstration of the importance of surveillance pathways in genome stability [28]. DSBs, which are among the most dangerous DNA lesions, are estimated to occur at a rate of ten per cell per day in primary human or mouse fibroblasts [29]. These naturally-occurring lesions are generated upon collapse of stalled DNA replication forks, replication across nicks, reactive oxygen species of endogenous origin or the untimely action of DNA endonucleases (topoisomerases or RAG and AID) [29]. Additionally, DSBs can occur as a result of lesions caused by external agents, such as ionizing radiation and mutagenic chemicals [8,30].

Despite the intense effort currently being exerted to identify proteins and pathways involved in recognition of the various forms of DNA damage, we are only beginning to appreciate the function of DNA repair mechanisms, their cooperation and regulation. In particular, the hierarchy and coordination of post-translational modifications (PTMs) on recruitment, function and stability of DNA repair proteins at sites of damage represent new challenges in the field [20,31]. To advance our understanding, and based on previous evidence obtained in our laboratory [5,22-24], we undertook a study aimed at investigating the molecular mechanism that controls the function of Exonuclease-1 (EXO1), a common component of machineries processing stalled replication forks, DNA base mismatches and DSBs [19,32-35]. EXO1 plays a key role in processing damaged DNA structures to generate intermediates that are conveyed to error-free pathways of repair [36-38]. Specifically, in this work we addressed the mechanism controlling proteasome-mediated degradation of EXO1 in response to agents that stall DNA replication and lead to the formation of DSBs. In search of the pathway that controls EXO1 protein stability, we found that EXO1 is a target of sumoylation under unperturbed conditions and in response to DNA damage, extending the observations made in a proteome-wide screen that addressed the role of SUMO in protein quality control and reported EXO1 among the SUMO targets [39]. We found that interfering with sumoylation blocks EXO1 ubiquitin-mediated degradation. We could

reconstitute EXO1 sumoylation *in vitro* and establish that EXO1 is sumoylated in an UBC9-PIAS1/PIAS4-dependent manner *in vivo*. Furthermore, we found that EXO1 constitutively interacts with the SUMO protease SENP6 that regulates its protein level in the cell. We provide evidence that sumoylation and ubiquitylation of EXO1 occur sequentially in response to DNA damage. The former facilitates EXO1 recruitment to sites of damage, whereas the latter occurs on EXO1 bound to chromatin and allows control of DNA processing during resection by targeting EXO1 to degradation (Fig. 9). In this respect, we observed that over-expression of EXO1, likely mimicking the condition of cancer cells with up-regulated *EXO1* gene expression [40], led to increased DNA resection, mostly resulting in DNA breaks. Identification of sumoylation sites allowed us to reveal reduced rates of chromosomal aberrations in cells expressing the EXO1 SUMO-mutant compared to the wild-type protein.

In summary, these data indicate that high EXO1 protein level represents a threat to genome stability. Although the cellular ubiquitylation machinery might still be able to cope with such a high level of EXO1, sumoylation-induced localization of this population to DNA eventually turns resection from physiological to pathological. Hence, we speculate that cancer cells with up-regulated *EXO1* gene expression ([40] and http://www.nextbio.com) possibly exploit inefficient regulation by SUMO pathways [20,41] to limit pathological resection of DNA, thus suppressing apoptotic signals that result from the accumulation of chromosomal aberrations of the type that we report. Future studies are needed to address this issue.

MATERIALS AND METHODS

Plasmids and constructs - pGFP-EXO1 was previously described [23]. Single- and multiple-point mutations were introduced in pEGFP-EXO1 and pTXB1-EXO1 by site-directed mutagenesis using Phusion DNA polymerase (Finnzymes/Thermo Scientific) and primers described in Table S1. pcDNA3.1-HA-ubiquitin, pCMV4-HA-UBC9, pCMV-Flag-SENP6 and pET28a-His-SENP6 (628-1112) were purchased at Addgene (Cambridge, MA, USA). pCMV-Myc-SUMO1 and pCMV-Myc-SUMO2 were kindly provided by P. Macchi (CIBIO, University of Trento, Italy). pET23a-His-RanGAP-tail was kind gift of F. Melchior (University of Heidelberg, Germany). The human E2 Ubiquitin Conjugating Enzyme siRNA library was purchased from Qiagen.

Antibodies and chemicals - The antibodies used in this study were either previously described [23] or purchased from NeoMarkers (mouse monoclonal anti-EXO1); Abcam (rabbit polyclonal anti-GFP, ab290); Calbiochem (mouse monoclonal anti-RPA2); Cell Signaling Tech. (rabbit monoclonal anti-γH2AX, anti-CHK1-pS345 and anti-PIAS4); Sigma (mouse monoclonal anti-β-tubulin and anti-FLAG); Upstate Biotech. Inc. (mouse monoclonal anti-γH2AX); S. Cruz Biotech. (rabbit polyclonal anti-TFIIH and mouse monoclonal anti-UBC9, anti-HA and anti-Myc tags). Monoclonal antibodies to SUMO1 (21C7) and SUMO2 (8A2) were purchased at DSHB, Iowa.

Secondary HRP-conjugated anti-mouse and anti-rabbit antibodies were form GE-Healthcare. Alexa Fluor-488, -594 and -647 conjugated secondary antibodies were from Invitrogen.

Camptothecin (Sigma), aphidicolin (Sigma), anacardic acid (Millipore) and PYR41 (Millipore) were dissolved in DMSO at 10 mM stock concentration. Hydroxyurea (Sigma) was dissolved in water at 1 M stock concentration. N-ehtylmaleimide (Sigma) was disolved in ethanol at 1 M stock concentration. MG-132 (Calbiochem) was prepared as 10 mM stock solution in DMSO and added to cells at 10 μM final concentration 30 min before additional treatments.

Preparation of 96-well library plates with pooled siRNAs and transfection mix and siRNA transfection - 96 wells contained a pool of 4 single siRNAs targeting one gene (final concentration = 20 nM for each single siRNA). siRNAs were diluted in Optimem and mixed with transfection reagent (Invitrogen, Lipofectamine 2000, 0.1 µl in 9.9 µl of Optimem per well). The cells were plated on top of the siRNA mix for a reverse transfection at a concentration of 2,000 cells/well in 80 µl of complete medium per well (DMEM +10%FCS). After 48 hrs of growth in complete medium to allow efficient knockdown of the targeted genes, cells were either left untreated or treated with HU for 16 hrs and then used for the image-based assay. Using an automated-systems for liquid handling, the medium was removed from each well and cells were washed twice in PBS, permeabilized for 5 min with 0.1% Triton-X100 at RT and stained with a mouse monoclonal antibody against the GFP-tag to amplify EXO1 signal. DAPI was used to stain nuclei.

High-content single-cell imaging of populations of cells - 96-well plates were imaged with a wide-field 20x objective microscope (ImageExpress Micro, Molecular Devices). Five focal planes per image and 49 sites per well were acquired. The maximum intensity projection of the 5 focal planes was saved for each site and used for further analysis. Images collected in the screen were stored as 16-bit uncompressed TIFFs.

Image analysis pipeline, single-cell feature - For each single cell, nuclei were detected based on the signal of the DAPI staining using the open-source software, Cell Profiler [42]. For every nucleus, the intensity for DAPI (blue) and EXO1 (green) (11 features per object and channel), shape (30 features per object) and texture features (15 features per object and channel) were extracted. For measurements of the population context of each single cell a point-spread function was used to measure the local cell density, the position of the cell in an islet (being on the edge or inside) and distance to cell islet edge (minimal distance between a cell and the edge of an islet) [43]. In total ~50 features per single cell were extracted.

Finally, for the single-cell EXO1 readout we z-scored (subtracting the average value over all single cells in the plate from each single-cell value and dividing this with the standard deviation of all single cells of the plate) the mean intensity per cell.

Protein expression and purification - Recombinant wild-type or mutant forms of EXO1 and the catalytic domain of SENP6 (628-1112) were expressed and purified as described in [5] and [44], respectively. The yeast SUMO machinery proteins (GST-Aos1/Uba2, His-Ubc9, His-Flag-Smt3, His-Flag-Smt3-KR, His-Siz1 (1-465), and Siz2) were purified as described [45,46]. Expression of human SUMO machinery from plasmids pET23a-UBC9, pET11a-SUMO1(1-97), and pET11a-SUMO2 (a kind gift from Frauke Melchior) was performed as described [47]. RanGAP-tail was purified as described [48].

The *S. cerevisiae* Exo1 protein with a C-terminal intein tag was expressed in *E. coli* BL21(DE3)-RIPL cells. After the cells reached OD_{600}~ 0.6, protein expression was induced by IPTG (1mM) for 24 h at 12°C. The cell pellet (18 g) was resuspended in 150 ml of cell breakage buffer (50 mM Tris-HCl pH 7.5, 10% sucrose, 10 mM EDTA, 1 mM β-mercaptoethanol, 0.01% Nonidet P-40) containing 100 mM KCl and protease inhibitors. Suspensions were sonicated and cleared by ultracentrifugation. The supernatant was loaded onto a 20 ml SP-Sepharose column. The column was developed with 200 ml gradient of 125–1000 mM KCl in buffer K (20mM KH_2PO_4, 10% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.01% Nonidet P-40). Peak fractions were pooled, diluted to 100 mM KCl and loaded onto a Heparin column (1 ml). The column was developed with 15 ml gradient of 150–1000 mM KCl in buffer K (20 mM KH_2PO_4, 10% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.01% Nonidet P-40). Peak fractions were pooled, concentrated, frozen in liquid N_2 and stored at -80°C.

The plasmid pGEX-4T-SAE2/SAE1 (a kind gift from Ronald T. Hay) expressing human E1 enzyme was introduced into *Escherichia coli* strain BL21(DE3). Protein expression was induced by 1 mM IPTG at 37°C for 4 h. The cell pellet (20 g) was resuspended in 70 ml of CBB buffer (50 mM Tris-HCl, pH 7.5, 10% sucrose, 2 mM EDTA, 150 mM KCl, 0.01% NP40, 1 mM

DTT, protease inhibitor cocktail), sonicated and cleared by ultracentrifugation. The resulting supernatant was applied onto a 7-ml Q-Sepharose column (GE Healthcare). The column was developed with 70 ml gradient of 100-900 mM KCl in buffer K (20mM KH_2PO_4, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40). Peak fractions were pooled and incubated with 1.5 ml Glutathione Sepharose 4B beads (GE Healthcare) for 1 hour at 4°C. The beads were washed with 20 ml of buffer K containing 100 mM KCl and proteins were eluted in steps with 10-100 mM glutathione in buffer K. The fractions containing GST-SAE2/SAE1 were applied onto a 1-ml MonoQ column (GE Healthcare), and eluted using 100-800 mM KCl in buffer K. The peak fractions were concentrated to 15 µg/µl in a Vivaspin-2 concentrator.

Cell culture and transfections - HEK293, HEK-293T and U2OS cells were maintained as described [23]. U2OS (kindly provided by S. P. Jackson, University of Cambridge, UK) and HEK293 cells stably expressing GFP-HA-EXO1 were cultured in DMEM supplemented with 10% fetal calf serum, standard antibiotics and G-418 (0.5 mg/ml) [5]. Transient transfections were performed using Truefect-Lipo (United Biosystems Inc.). siRNA oligonucleotides (Microsynth) used in this study are listed in Table S1. siRNA duplexes were transfected at 40 nM concentration using Truefect-Lipo (United BioSystems Inc. USA) in two consecutive rounds. Experiments were typically performed 48-72h.

Pull-down, immunoprecipitation and Western blotting - Purified SENP6 protein was incubated with 25 µl of intein-tagged EXO1 pre-bound to chitin beads (New England Biolabs) for 30 min at 4°C with gentle shaking in 25 µl of buffer T (25 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 10% glycerol) containing 100 mM KCl. After incubation, the supernatants (S) were collected and 20 µl SDS Laemmli buffer was added. Beads were washed with 100 µl of buffer T and bound proteins were eluted with 30 µl of SDS Laemmli buffer (eluate, E). S and E fractions were analyzed by SDS-PAGE.

To assess ubiquitylation or sumoylation *in vivo*, cells expressing HA-ubiquitin or Myc-SUMO1/Myc-SUMO2 were lysed in buffer B (50 mM Tris-HCl pH 7.5, 5 mM dithiothreitol, 0.5 mM N-ethylmaleimide (NEM), 1% SDS) and incubated

for 10 min at 95°C. Samples were sonicated and clarified by centrifugation for 10 min at maximum speed in an Eppendorf centrifuge. Finally, samples were diluted with four volumes of buffer A (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na-pyrophosphate, 0.5 mM Na-orthovanadate, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40) prior to immunoprecipitation.
Immunoprecipitation and immunoblot analysis were performed as described previously [24] and using the FUSION SOLO® chemiluminescence imaging system (Vilber). Ethidium bromide was present in co-immunoprecipitation experiments to rule out DNA-mediated interactions.

Immunofluorescence staining and analyses - Cells grown on cover slips were either fixed directly in ice-cold methanol for 15 min or pre-extracted for 5 min on ice using 25 mM HEPES pH 7.4, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose and 0.5% Triton X-100 before fixation in 4% formaldehyde (w/v) in PBS for 15 min at room temperature (RT). Cover slips were incubated overnight at 4 °C with primary antibodies and Alexa–conjugated secondary antibodies for 1h at RT. The cover slips were mounted with Vectrashield® (Vector Laboratories) containing DAPI. Images were acquired using a Leica fluorescence microscope.

***In vitro* sumoylation assay** - The *in vitro* sumoylation assay using yeast proteins was performed in a 10 µl volume containing 150 nM Aos1/Uba2, 250 nM Ubc9, 4.3 µM Smt3-KR, 10 nM Siz1 or Siz2, 1 mM ATP, buffer S1 (100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$), and 1 µM human or yeast Exo1. Reactions were incubated at 30°C for 45 min, stopped by addition of SDS Laemmli buffer and analyzed by SDS-PAGE.
The *in vitro* sumoylation assay using human proteins was performed in a 10 µl volume containing 100 nM GST-SAE2/SAE1, 2.8 µM UBC9, 4.3 µM SUMO1, 4.3 µM SUMO2, 1 mM ATP, buffer S (50 mM HEPES, 10 mM $MgCl_2$, 0.1 mM DTT), and 1 µM EXO1. Reactions were incubated at 30°C for 1h, stopped by addition of SDS Laemmli buffer, and analyzed by SDS-PAGE.

Identification of sumoylation sites by LC-MS/MS - SDS-PAGE protein bands were reduced with dithiothreitol (DTT), alkylated by incubation with iodoacetamide and subsequently digested by addition of proteomics grade trypsin (Roche) over night at 37°C. Digests were separated on an UltiMate 3000 RSLCnano (Dionex/Thermo Fisher Scientific) with a trapping column (PepMap C18, 5µm particle size, 300 µm i.d. x 5mm, Dionex/Thermo Fisher Scientific) equilibrated with 0.1% TFA and an analytical column Acclaim PepMap RSLC C18, 50 cm × 75 µm × 2 µm, 100 Å, Dionex/Thermo Fisher Scientific) applying a 1.6% to 30% acetonitrile (ACN) linear gradient in 30 min. The HPLC was directly connected to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ionization source (Proxeon/Thermo Fisher Scientific) set to 2 kV. The mass spectrometer was operated in data-dependent mode: 1 full scan in the orbitrap (m/z: 350-2000, resolution 60000) with lock mass (m/z 445.120025) enabled was followed by maximal 6 HCD and 6 CID scans. Monoisotopic precursors were selected, singly charged signals were excluded from fragmentation. For CID, normalized collision energy was set at 35% or 30%, Q-value at 0.25 and the activation time at 10 ms. HCD parameters were 0.1 ms activation time and 35% normalized collision energy. Fragmented precursors were excluded from further selection for 30 s.

Peptide identification was performed either by Sequest or Mascot 2.1 (Matrix Science) through the Proteome Discoverer 1.4 or by MassMatrix 2.4.2 [49]. Spectra were searched against a small database containing protein sequences plus proteases and contaminants. Search parameters were: tryptic specificity with max. 4 missed cleavages, peptide tolerance of 5 ppm, fragment ions tolerance of 0.8 Da for CID, 0.05 Da for HCD spectra. Carbamidomethylation of Cys was set as static modification, oxidation of Met as a variable modification. For the Sequest and Mascot search, the peptide mass of the linked fragment EQIGG (+484.2218) from the SUMO protein was set as a variable modification of lysines. Alternatively proteins of interest were digested *in silico* with trypsin and the resulting peptides were extended N-terminally with the sequence of EQIGG and added to the database as described in [50]. For the search with MassMatrix, SUMO protein sequence was truncated at the last C-terminal glycine (..EQIGG) and this residue marked as

the cross-linkage site to lysine residues. All spectra assigned to branched peptides were validated manually.

DNA-binding and nuclease assays - The hairpin and 3‘-overhang substrates were prepared by annealing of 3‘ fluorescently labelled HL-1 oligonucleotide or oligo-1 and -3 (Table S1) (VBC Biotech) in hybridization buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) for 5 min at 70°C. The 3’flap substrate was prepared using Oligos-1, -2, and -3 (VBC Biotech) according to the procedure described [51].

For EMSA, indicated amounts of EXO1 protein were incubated with fluorescently labeled DNA substrate (4 nM) in 10 µl of buffer E (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 50 ng/µl bovine serum albumin) at 37°C for 10 min. Following the addition of loading buffer (60% glycerol, 10 mM Tris-HCl pH 7.4 and 60 mM EDTA), the samples were separated on native polyacrylamide gel (10%) in 0.5x TBE buffer (40 mM Tris-HCl pH 7.5, 20 mM boric acid, 2 mM EDTA). DNA was visualized by FLA9000 Starion (Fujifilm) and quantified using MultiGauge software (Fujifilm).

For nuclease assays, indicated amounts of EXO1 protein were incubated with fluorescently labeled DNA substrate (4 nM) in 10 µl of buffer EN (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/ml bovine serum albumin) at 37°C for 20 min. The reactions were stopped by adding SDS (0.05 %) and proteinase K (0.5 mg/mL) at 30°C for 3 min. After adding loading buffer (10 mM Tris-HCl pH 7.4, 60 mM EDTA, 60% glycerol), the samples were separated on native polyacrylamide gel (10%) in TBE buffer. DNA was visualized and quantified as described above.

Enrichment of chromatin-bound proteins - A Triton-based extraction protocol [52] was used to isolate insoluble proteins. Cells were washed twice in cold PBS and once in pre-extraction buffer (25 mM HEPES pH 7.4, 50 mM NaCl, 1mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose, 0,5% Triton X-100 and protease inhibitors), followed by incubation on ice for 5 min in pre-extraction buffer. Cells were then washed once with cold PBS and harvested by scraping into Laemmli buffer, heat denatured, sonicated and analyzed by Western blot.

Laser micro-irradiation of nuclei - DSBs in defined nuclear volumes were generated as described [5], using micro-irradiation (MMI CellCut) with a 355 nm UV-A laser adjusted at 50% of the power. Prior to irradiation, cells were grown for 24 h in the presence of 10 μM BrdU.

Flow cytometric analysis - To quantify DNA-end resection in response to CPT or HU, the extent of chromatin-bound RPA was assessed by flow cytometry. Briefly, cells either stably or ectopically expressing GFP-EXO1 were harvested, pre-extracted in 0.2% Triton X-100/PBS and fixed with 4% formaldehyde/PBS. Cells were washed with 1% BSA/PBS, permeabilized with 0.5% saponin/1% BSA/PBS, and stained with anti–γ-H2AX antibody (#9718; Cell Signaling Technology) and anti-RPA (#NA19L, Calbiochem) for 2 h, followed by incubation with a suitable Alexa-labeled secondary antibody for 30 min. DNA was stained with 1 μg/ml DAPI. Samples were measured on a Cyan ADP flow cytometer (Beckman Coulter) and analyzed with Summit software v4.3 (Beckman Coulter).

Chromosome analysis - Metaphase spreads were prepared as described in [5]. Briefly, after treatment with 2.5 μM camptothecin for 1h, cells were allowed to recover for 8h in complete medium before chromosome preparation. Caffeine (2 mM) was added for the last 5h to override the G2/M checkpoint, and colcemid (0.1 mg/ml) was added for the last 3h to arrest cells in metaphase. Metaphase chromosomes were stained with DAPI.

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FIGURE LEGENDS

Figure 1 - Camptothecin targets EXO1 for proteasome-mediated degradation

a. HEK-293T cells were treated with camptothecin (CPT, 1 μM), aphidicolin (APH, 15 μM) and MG-132 (10 μM) for 4h, as indicated. Endogenous EXO1 was immunoprecipitated with rabbit polyclonal antibody F15 and visualized with a specific monoclonal antibody. IgG(H) were used as control for the quality of the immunoprecipitation (IP). Whole cell extracts (WCEs, inputs) were analyzed before IP using the indicated antibodies.
b. HEK-293T cells ectopically expressing GFP-EXO1 were treated with increasing doses of CPT for 4h. WCEs were examined using the indicated antibodies.
c. HEK-293T cells ectopically expressing GFP-EXO1 (left) or stable U2OS-GFP-EXO1 cells (right) were treated with CPT (1 μM) and MG-132 (10 μM) for 4h, as indicated. WCEs were analyzed using the indicated antibodies. Mock transfected HEK-293T and wild-type U2OS were used as controls.
d. Indirect immunofluorescence analysis of wild-type and stable U2OS-GFP-EXO1 cells shown in c.
e. HEK-293T cells ectopically expressing GFP-EXO1 were treated with CPT (1 μM), MG-132 (10 μM) and the E1-UBI inhibitor PYR-41 (50 μM) for 4h, as indicated.

Figure 2 - UBC9 controls EXO1 protein level

a. UBC9 depletion causes stabilization of EXO1. An E2-ubiquitin conjugating enzymes siRNA library was screened on stable U2OS-GFP-EXO1 cells left untreated or treated with HU. Mean intensity of the green signal averaged over all cells per gene were plotted (left of the panel). Single cell distribution of the green signal for Ctrl and UBC9 (UBE2I in the figure) depleted cells were plotted (right of the panel). Entire well images and magnified indicative fields (greyscale) of Ctrl

and UBC9 siRNA-depleted cells are shown. Scale bar entire field = 350 μm. Scale bar enlargement = 50 μm.

b. Western blot analysis of wild-type or stable U2OS-GFP-EXO1 cells transfected with CTRL or UBC9 siRNA oligonucleotides and treated with CPT. WCEs were analyzed using the indicated antibodies.
c. Western blot analysis of HEK-293T cells ectopically expressing GFP-EXO1 that were treated and analyzed as in b.
d. Indirect immunofluorescence of stable U2OS-GFP-EXO1 cells treated as indicated in b. Image processing and analysis software (ImageJ) was used to calculate the percentage of green positive events from the DAPI stained cells examined (N).
e. UBC9 over-expression decreases EXO1 protein level. Western blot analysis of HEK-293T cells ectopically expressing GFP-EXO1 and HA-UBC9 that were treated with CPT (1 μM) for 4h. WCEs were analyzed using the indicated antibodies.

Figure 3 - *In vitro* reconstitution of EXO1 sumoylation

a. Yeast Exo1 is sumoylated *in vitro*. Sumoylation of yeast Exo1 was reconstituted *in vitro* with yeast E1, E2 and Smt3-KR in the presence or the absence of the E3-ligases Siz1 or Siz2. Samples were resolved by SDS-PAGE and visualized by silver staining. Asterisks indicate the sumoylated forms of Exo1.
b. Human EXO1 is sumoylated *in vitro*. *In vitro* sumoylation of human EXO1 was performed as described in a. Samples were resolved by SDS-PAGE and visualized by silver staining. Asterisks indicate the sumoylated forms of EXO1.
c. Recombinant Ran-GAP-tail was used as model substrate to assess the functionality of the *in vitro* reconstituted human sumoylation machinery. Samples were resolved by SDS-PAGE and visualized by Western blotting.
d. EXO1 sumoylation with a reconstituted human SUMO-machinery. *In vitro* sumoylation of human EXO1 was performed with human E1, E2, SUMO1 and SUMO2. Samples were resolved by SDS-PAGE and

visualized by silver staining or Western blotting using a monoclonal antibody to SUMO1. Asterisks indicate the sumoylated forms of EXO1.

Figure 4 - EXO1 is target of sumoylation *in vivo*

a. HEK-293T cells ectopically expressing MYC-SUMO1, MYC-SUMO2 and GFP-EXO1 were treated with HU (2 mM) and MG-132 (10 μM) for 15h, as indicated. GFP-EXO1 was immunoprecipitated with a rabbit polyclonal antibody to GFP and visualized using a monoclonal antibody to the MYC-tag. The membrane was stripped and re-probed with a mouse monoclonal to GFP. IgG(H) were used as control for the quality of the IP. WCEs (inputs) were analyzed before IP using the indicated antibodies.
b. EXO1 is sumoylated in a SUMO1-dependent manner. Western blot analysis of HEK-293T cells ectopically expressing MYC-SUMO1 or MYC-SUMO2 and treated with CPT. WCEs were analyzed using the indicated antibodies.
c. PIAS1/PIAS4 depletion increases EXO1 protein stability. HEK-293T cells ectopically expressing GFP-EXO1 were depleted for PIAS1 or PIAS4 and treated with CPT. WCEs were analyzed using the indicated antibodies.
d. Depletion of SENP6 affects EXO1 protein level. Western blot analysis of stable U2OS-GFP-EXO1 cells depleted for SENP5 or SENP6. WCEs were analyzed using the indicated antibodies.
e. EXO1 interacts with SENP6 *in vivo*. HEK-293T cells were transfected with Flag-SENP6 and GFP-EXO1 as indicated and treated with CPT. WCEs were immunoprecipitated with a mouse monoclonal antibody to the Flag and membranes were probed with a rabbit polyclonal antibody to GFP. The membrane was stripped and re-probed with a mouse anti-Flag monoclonal antibody. IgG(H) were used as control for the quality of the immunoprecipitation (IP). WCEs were analyzed before IP using the indicated antibodies.
f. EXO1 and SENP6 interact *in vitro*. Intein-tagged EXO1 was bound to chitin beads and used as prey to capture purified recombinant SENP6

protein (628-1112 aa). The silver-stained gel shows proteins remaining in the supernatant after capture (S) or elution from chitin beads (E) trapping intein-EXO1 (lanes 1 and 2) or beads alone (lanes 3 and 4). The position of EXO1 and SENP6 is indicated.

Figure 5 - Sumoylation and ubiquitylation occur sequentially on EXO1

a. Chemical inhibition of SUMO-pathways impairs EXO1 ubiquitylation. Wild-type and stable U2OS-GFP-EXO1 cells were transiently transfected with HA-ubiquitin and left untreated or treated with the E1-SUMO inhibitor anacardic acid (5 mM), the E1-UBI inhibitor PYR-41 (50 mM) or the proteasome inhibitor MG-132 (10 mM). CPT was used as DNA damaging agent. WCEs were immunoprecipitated with a rabbit polyclonal antibody to GFP and membranes were probed with a monoclonal antibody to the HA tag. The membrane was stripped and re-probed with a mouse monoclonal to GFP. WCEs were analyzed before IP using the indicated antibodies.
b. UBC9 depletion blocks EXO1 ubiquitylation. Stable U2OS-GFP-EXO1 cells were transfected with CTRL or UBC9 siRNA oligonucleotides and treated with CPT. WCEs were immunoprecipitated and analyzed as in a.

Figure 6 - Sumoylation controls EXO1 recruitment to DNA

a. Sumoylation does not affect DNA binding of EXO1 *in vitro*. Increasing concentrations of non-sumoylated or sumoylated EXO1-D173A were tested for DNA binding activity using a 3'-flap substrate (4 nM). Quantification of the gel using MultiGauge software is shown.
b. UBC9 depletion affects EXO1 recruitment at sites of damage. Stable U2OS-GFP-EXO1 cells transfected with CTRL or UBC9 siRNA oligonucleotides were laser microirradiated and fixed at the indicated time points. Cells were immunostained with antibodies to γH2AX and GFP and analyzed by fluorescence microscopy. Nuclei were visualized with DAPI.
c. Western blot analysis of the cells shown in b.

d. Chromatin-bound EXO1 is ubiquitylated. HEK-293T cells ectopically expressing GFP-EXO1 were treated with CPT and MG-132. GFP-EXO1 present in the soluble and the chromatin fractions were detected by Western blot.
e. UBC9 depletion decreases the ratio of chromatin-bound to free EXO1. HEK-293T cells ectopically expressing GFP-EXO1 were transfected with CTRL or UBC9 siRNA oligonucleotides. The total and chromatin-bound fractions of GFP-EXO1 were detected by Western blot. Established markers for the total or the chromatin fraction were used.

Figure 7 - EXO1 is sumoylated on lysine residues $K_{655/801/802}$

a. Schematic representation of EXO1. The N- and I-catalytic domains are shown in orange and the five potential sumoylation sites are shown in blue. Amino acid sequences flanking sumoylation sites are shown.
b. EXO1-3KR is not sumoylated *in vitro*. *In vitro* sumoylation assay of human EXO1-wt or EXO1-3KR ($K_{655,801,802}$>R) was performed with yeast E1, E2, Smt3-KR and Siz1 in the presence or the absence of ATP. The asterisk indicates the sumoylated form of EXO1.
c. EXO1-3KR possesses DNA binding activity similar to that of EXO1-wt. The DNA binding activity of purified EXO1-wt or EXO1-3KR was assessed using a 3'-flap substrate. Quantification of the gel using MultiGauge software is shown at the bottom.
d. *In vitro* exonuclease assay. The exonuclease activity of purified EXO1-wt or EXO1-3KR was assessed using a hairpin substrate. Quantification of the gel using MultiGauge software is shown at the bottom.

Figure 8 - Defective EXO1 sumoylation reduces chromosomal aberrations caused by camptothecin

a. Sumoylation does not affect EXO1 nuclease activity *in vitro*. Increasing amounts of non-sumoylated and sumoylated EXO1 were assessed for nuclease activity on a hairpin substrate (4 nM). Quantification of the gel using MultiGauge software is shown at the bottom.

b. Extent of *in vitro* sumoylation of EXO1 used in the nuclease assay described in a.
c. EXO1 protein level in the cell affects the extent of DNA resection. The extent of chromatin-bound RPA as indicator of DNA resection was assessed by flow cytometric analysis in U2OS cells ectopically expressing GFP-EXO1.
d. Western blot analysis of the cells described in c.
e. Sumoylation sites mutation rescues the chromosomal aberrations caused by EXO1. Chromosome breaks observed in metaphase spreads of HEK-293T cells ectopically expressing GFP-EXO1-wt or GFP-EXO1-3KR and treated in the presence or the absence of CPT. Inset: example of chromosome breaks used in the quantification.
f. Western blot analysis of the cells described in e.

Figure 9 - Model of the sequential modifications of EXO1

Reversible EXO1 sumoylation is controlled by UBC9-PIAS1/PIAS4 and SENP6. In response to stalled replication, sumoylated EXO1 is recruited to DNA and subsequently ubiquitylated. Proteasome-dependent degradation ensues, thus controlling the extent of DNA resection and avoiding chromosomal aberrations.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1 (related to Fig. 2) - RNAi-based screening of human E2 ubiquitin conjugating enzyme library

Distribution of local cell density (cell crowding), nuclear size and total intensity of the DAPI staining (indicative for cell cycle phases) were plotted for Ctrl and UBC9 (UBE2I) siRNA-depleted cells left untreated or treated with HU.

Supplementary Figure 2 (related to Fig. 2) - Quantification of green fluorescence intensity in stable U2OS-GFP-EXO1 cells depleted for UBC9

a. IF images from siCTRL and siUBC9 cells. Left: merge of DAPI (DNA) and GFP (EXO1) signals. Middle: DAPI images converted to binary colors. Right: EXO1-positive cells identified over the threshold applied.
b. Quantification of the EXO1 signal intensity. The green fluorescent signal in siCTRL and siUBC9-positive cells from panel A was quantified and values were normalized to siCTRL cells.

Supplementary Figure 3 (related to Fig. 4) - Efficiency of SENP6 depletion

Western blot analysis of HEK-293T WCEs upon depletion with CTRL or SENP6 siRNA oligonucleotides.

Supplementary Figure 4 (related to Fig. 7) - Identification of sumoylation sites in EXO1

a. LC-MS/MS identification of SUMO-K_{655}. Annotated CID spectrum of the +5-charged branched peptide KSDSPTSLPENNM(ox)SDVSQLK(sumo)SEESSDDESHPLR proving SUMOylation at K655. b-ions are indicated in red and y-ions in blue. The peptide was identified with Sequest with an XCorr value of 4.47 and shows a mass deviation of 0.23 ppm.
b. LC-MS/MS identification of SUMO-K_{801}. Annotated CID spectrum of the doubly charged branched peptide NFGFK(sumo)K showing

SUMOylation at K801. The assigned b-ions are indicated in red and y-ions in blue. The fragment ions `y and `b result from the dissociation along the sumo-side chain (EQIGG) and are indicated in green. The peptide was identified with MassMatrix with a Score of 44 and 0.0018 Da mass deviation.

Supplementary Figure 5 (related to Fig. 7) - Nuclease activity of EXO1-5KR

In vitro exonuclease assay. The exonuclease activity of purified EXO1-wt or EXO1-5KR was assessed using a 3'-overhang substrate.

Supplementary Figure 6 (related to Fig. 8) - Analysis of DNA resection by flow cytometric analysis

Quantification of DNA resection in HeLa cells. Quantitative flow cytometric analysis of DNA end resection (RPA) and DNA content (DAPI) in HeLa cells ectopically expressing GFP-EXO1-wt and treated with HU.

Supplementary Figure 7 (related to Fig. 8) - Metaphase spreads

Representative images of metaphase spreads. Mock transfected HEK-293T and cells ectopically expressing GFP-EXO1-wt or GFP-EXO1-3KR were treated in the presence or the absence of CPT and metaphase spreads prepapred as described in Materials and Methods. Examples of chromosome breaks used in the quantification are indicated by arrows.

Supplementary Table 1 - List of oligonucleotides used in RNA interference studies, nuclease assays and generation of point mutations in EXO1, respectively.

siCTRL	CGUACGCGGAAUACUUCGATT
siUBC9	CCACCAUUAUUUCACCCGATT
siEXO1	CAAGCCUAUUCUCGUAUUUTT
siPIAS1	CGAAUGAACUUGGCAGAAATT
siPIAS4	AGGCACUGGUCAAGGAGAATT
siSENP6	AAGGCGUAUGUAUUAAGUAAATT
HL-1	ATCATTGCCTATCCTGACAGTCCGACACATCGGACTGTCA GG ATAGGCAATGATCTTTTTTTTT
Oligo-1	AGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATG GTCATAGCT
Oligo-2	AGCTATGACCATGATTACGAATTGCTTGGAATCCTGACGAA CTGTAG
Oligo-3	AATTCGTGCAGGCATGGTAGCT
EXO1(K_{655}R)-for	GTGTCGCAGTTAAGGAGCGAGGAGTCC
EXO1(K_{655}R)-rev	GGACTCCTCGCTCCTTAACTGCGACAC
EXO1($K_{801/802}$R)-for	GGAAAAACTTTGGATTTAGAAGAGATTCTGAAAAGC
EXO1($K_{801/802}$R)-rev	GCTTTTCAGAATCTCTTCTAAATCCAAAGTTTTTCC

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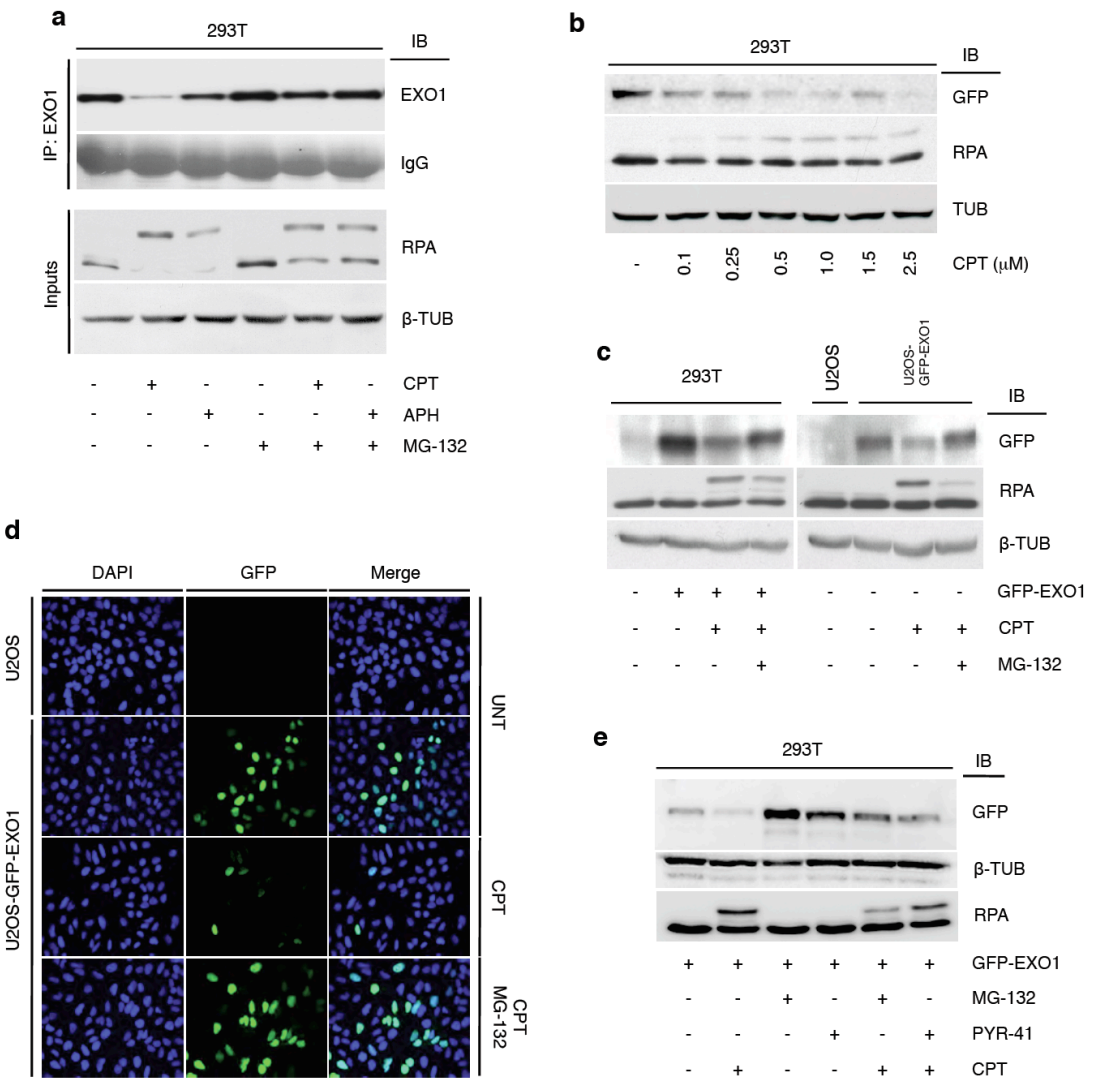

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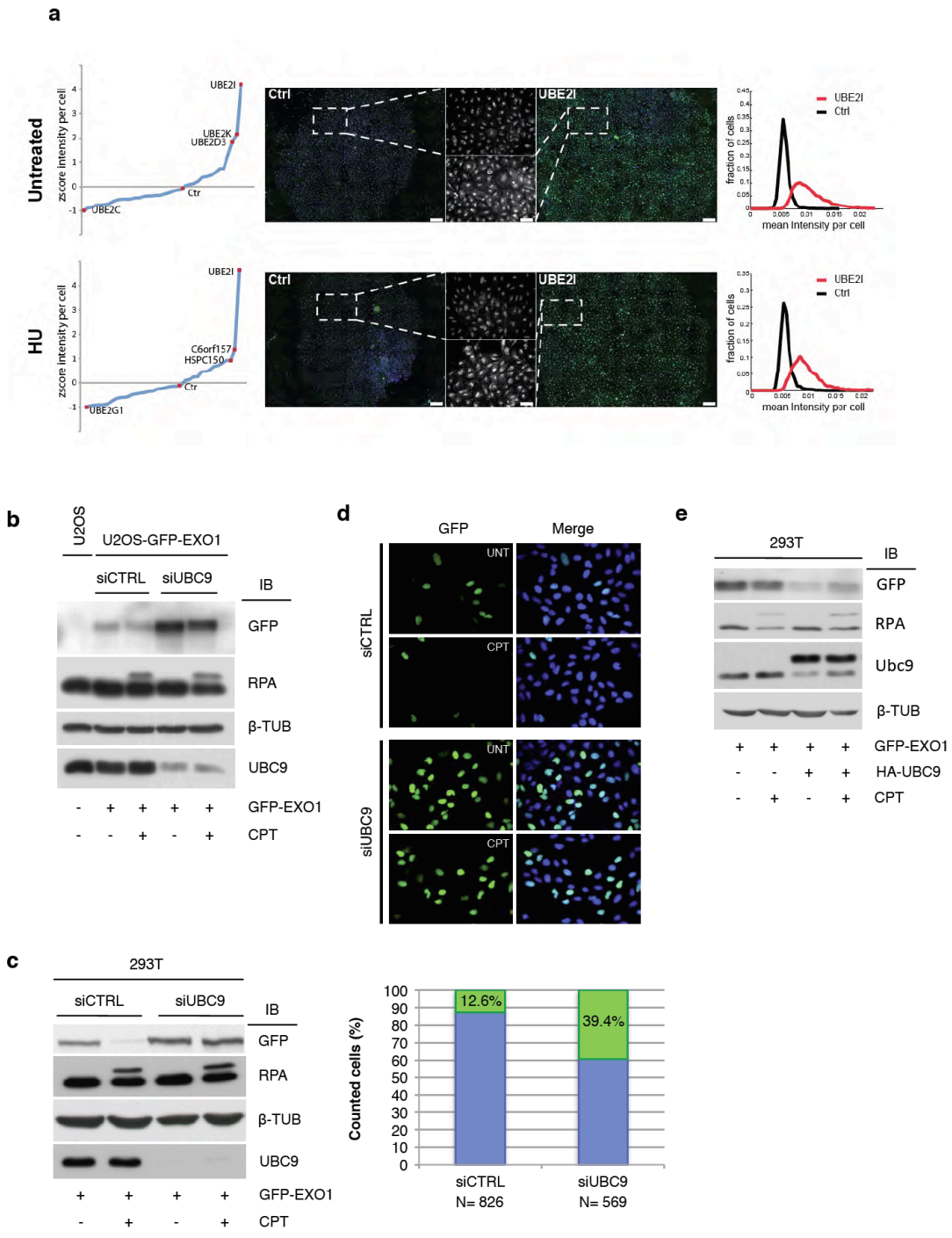

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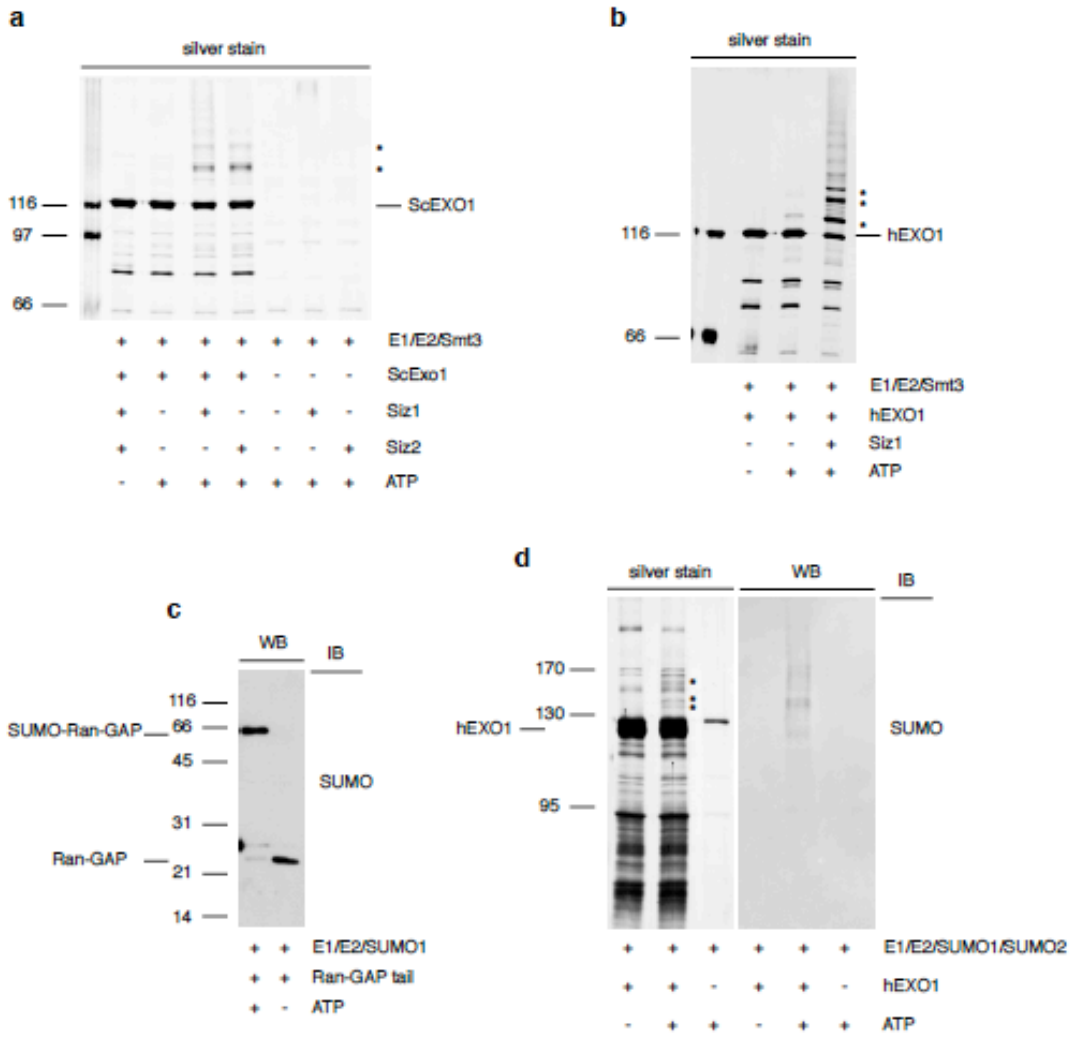

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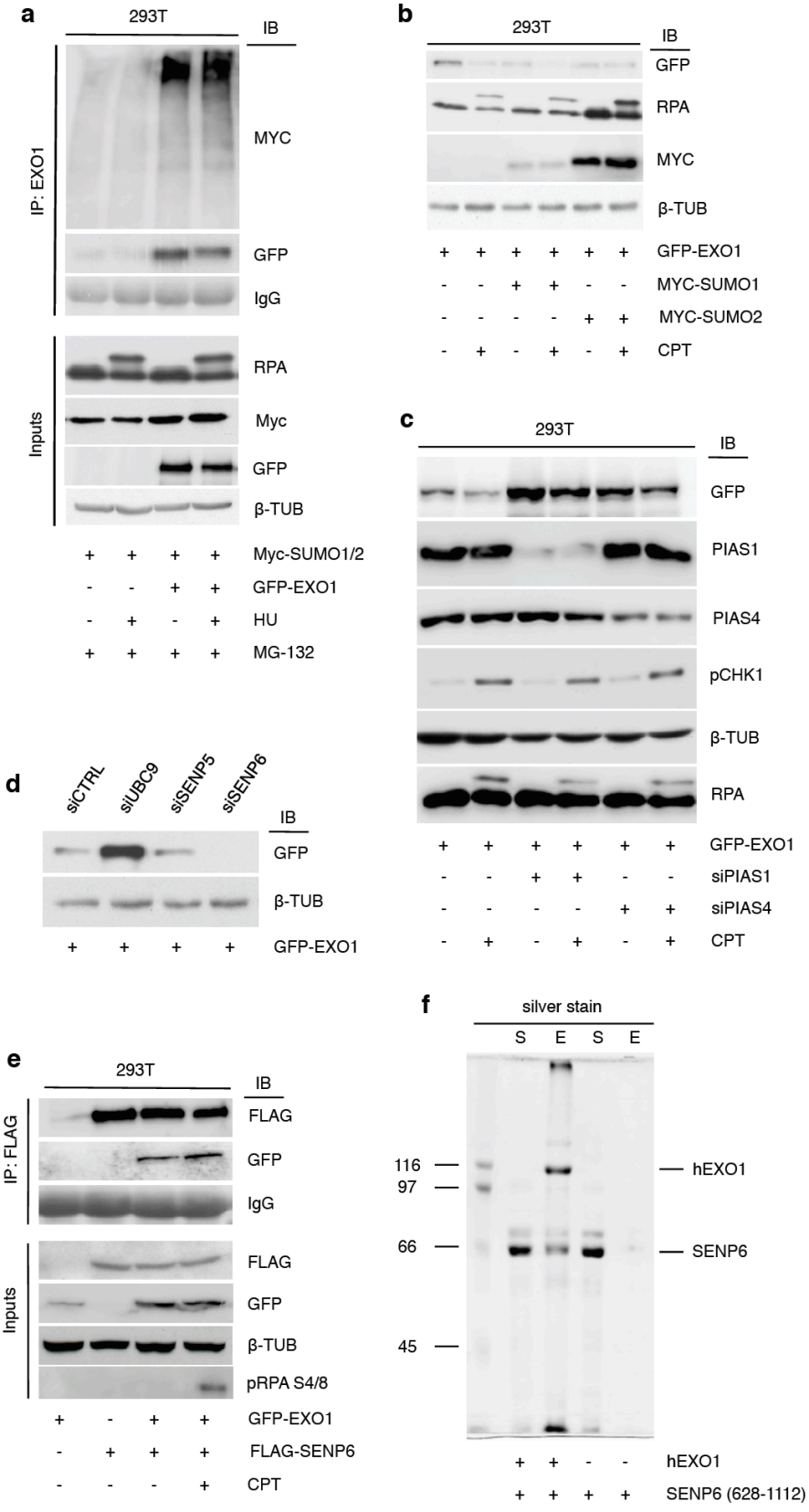

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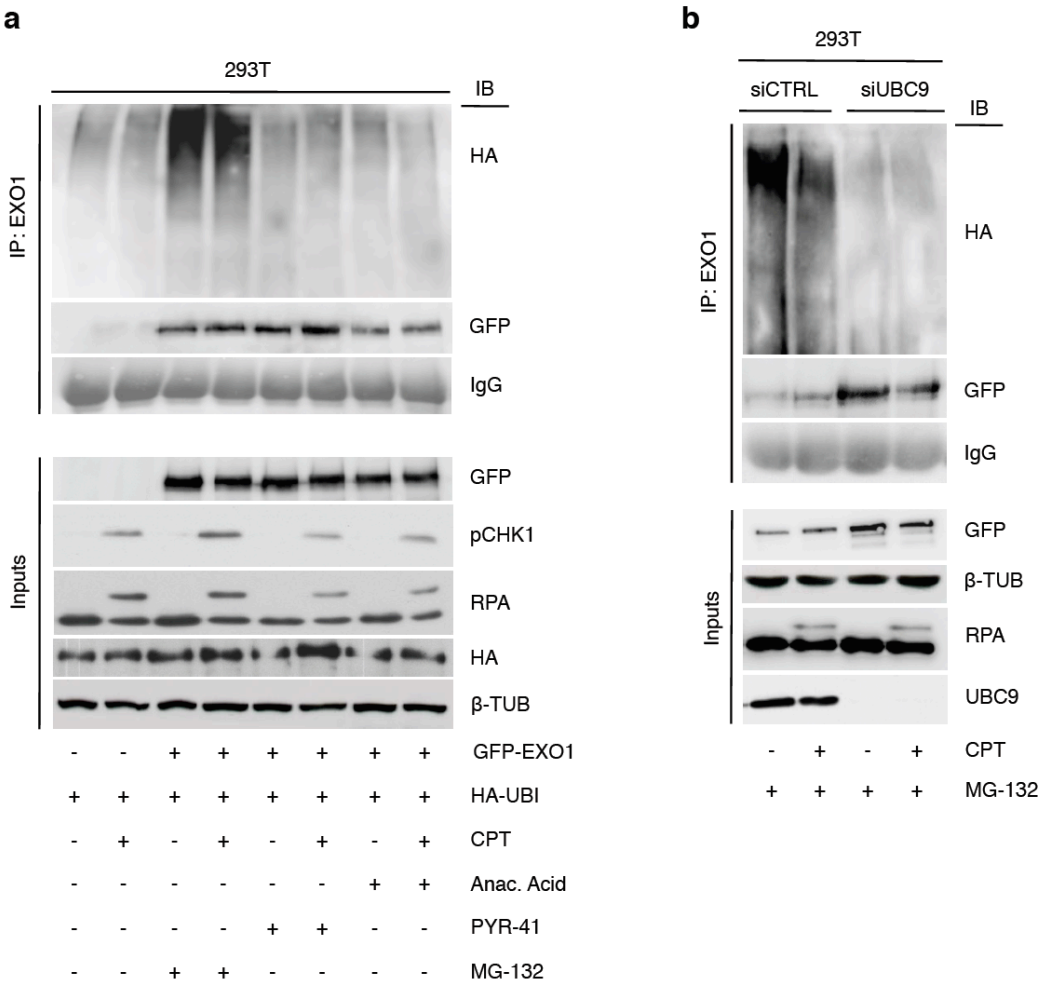

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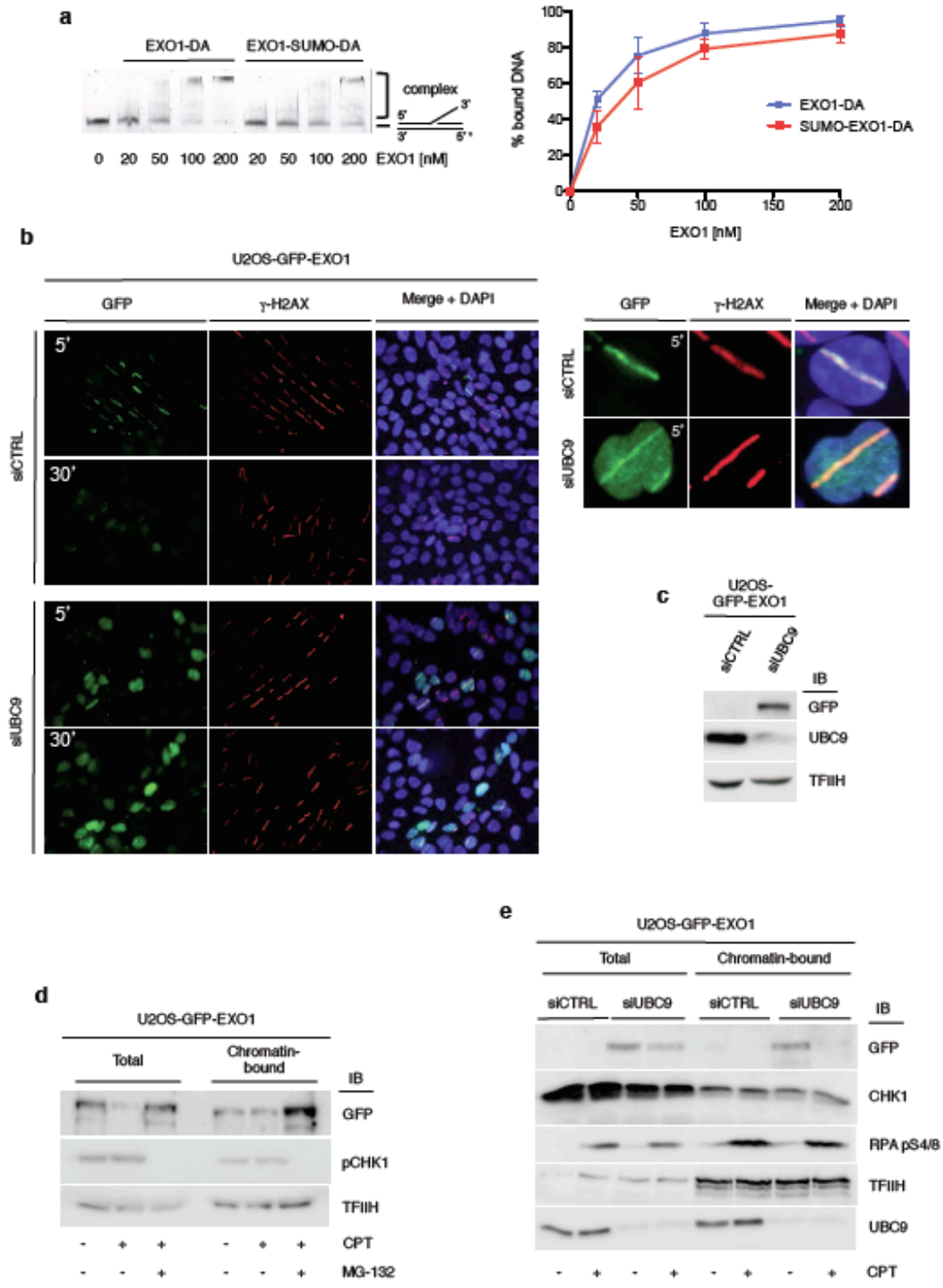

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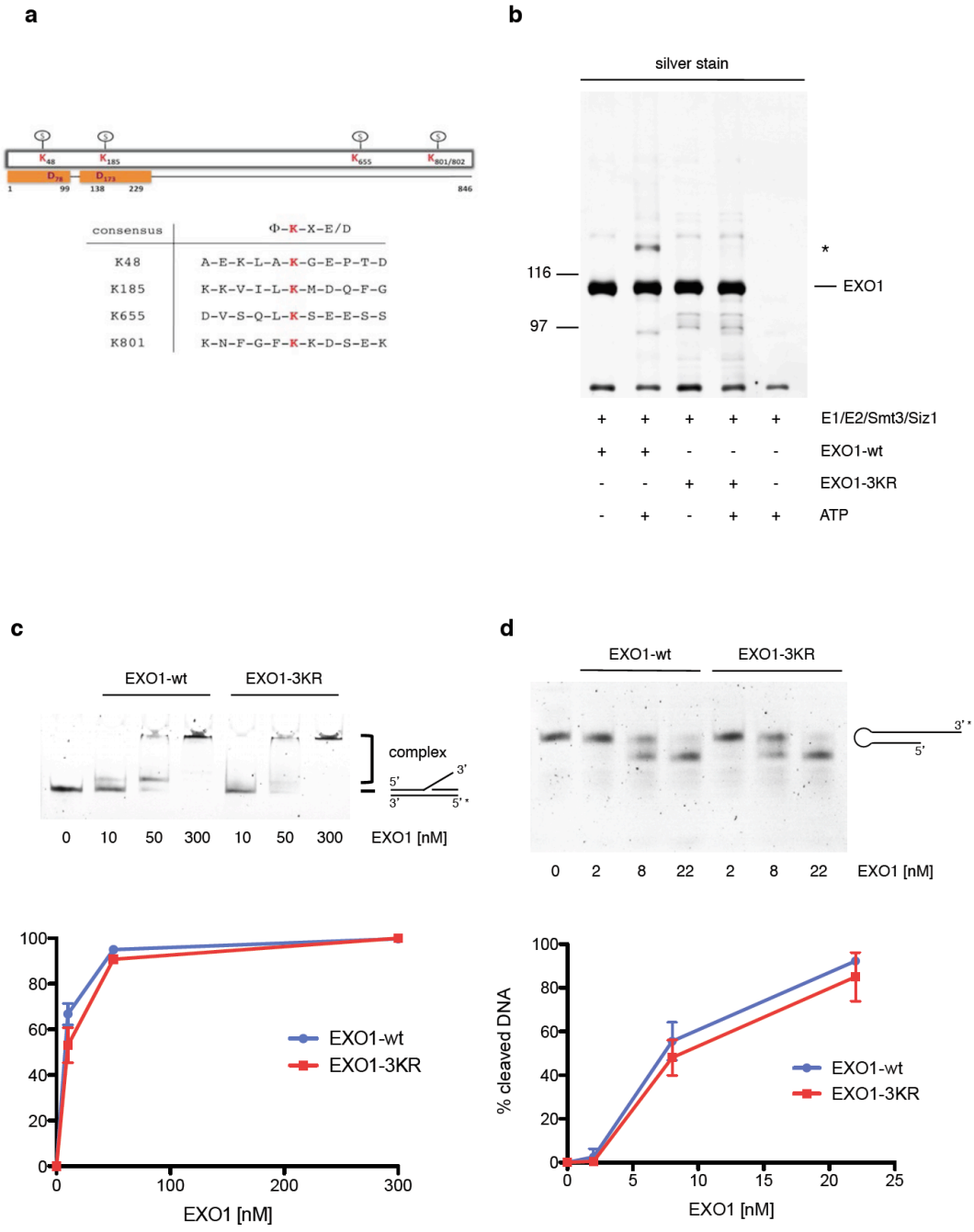

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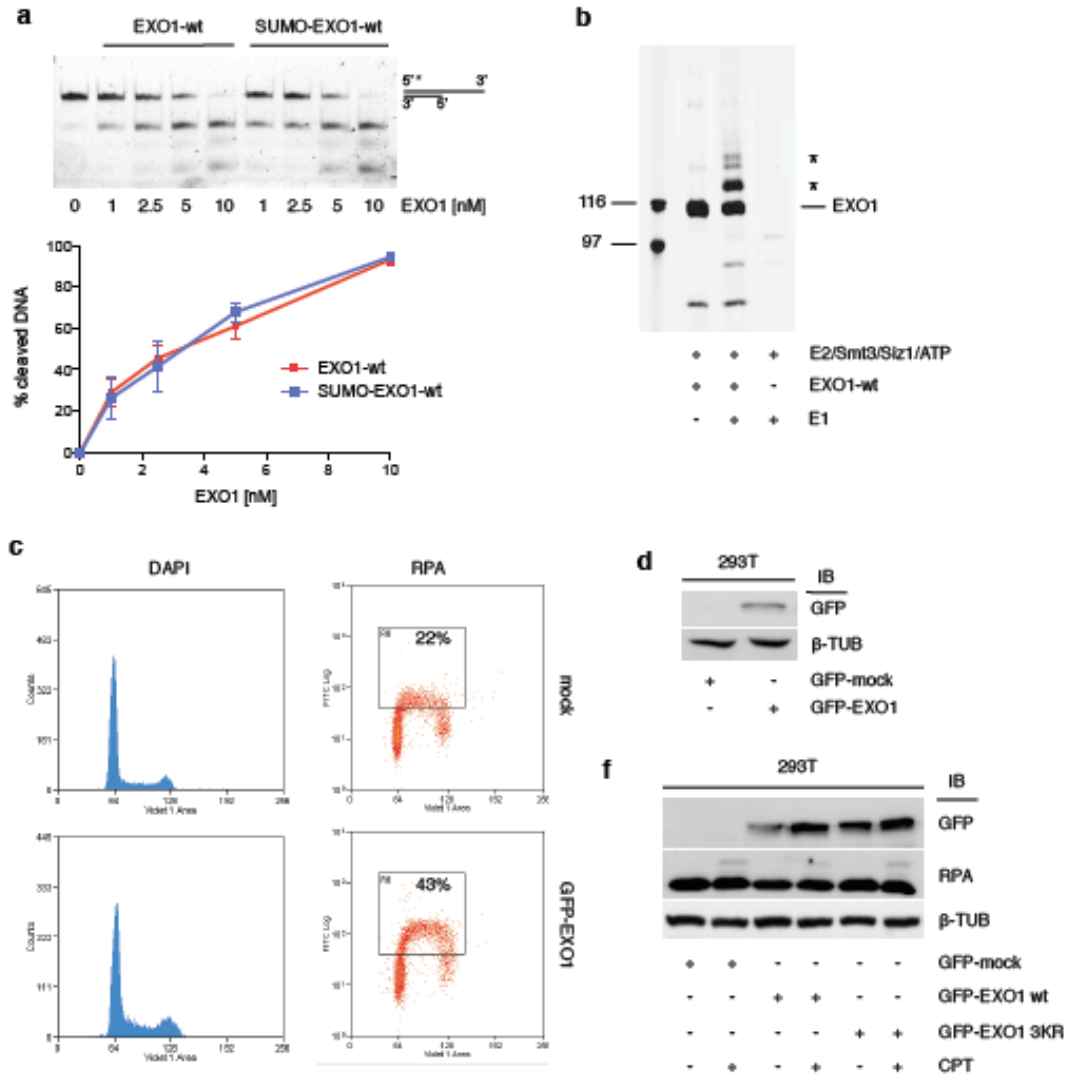

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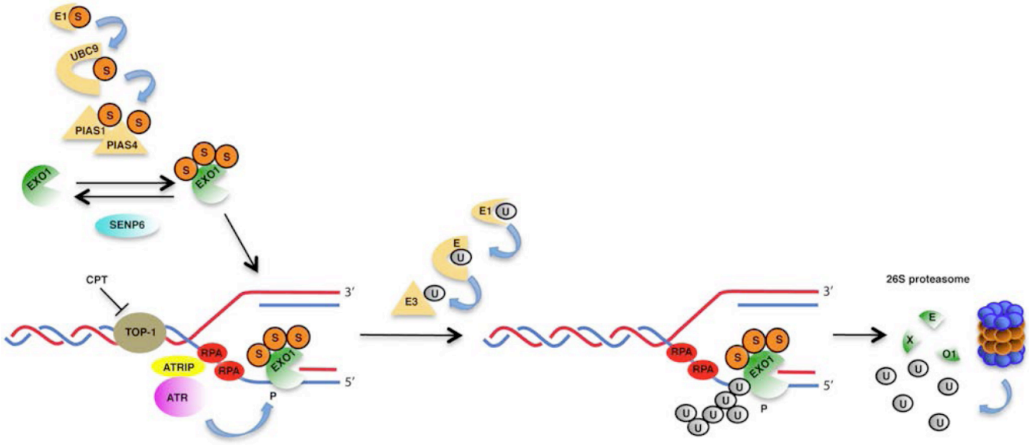

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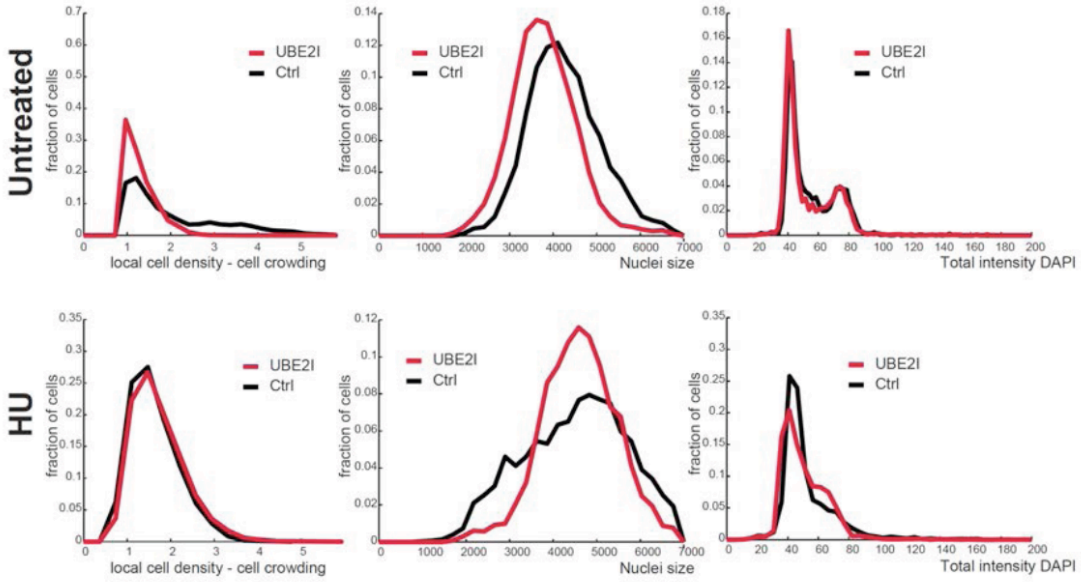

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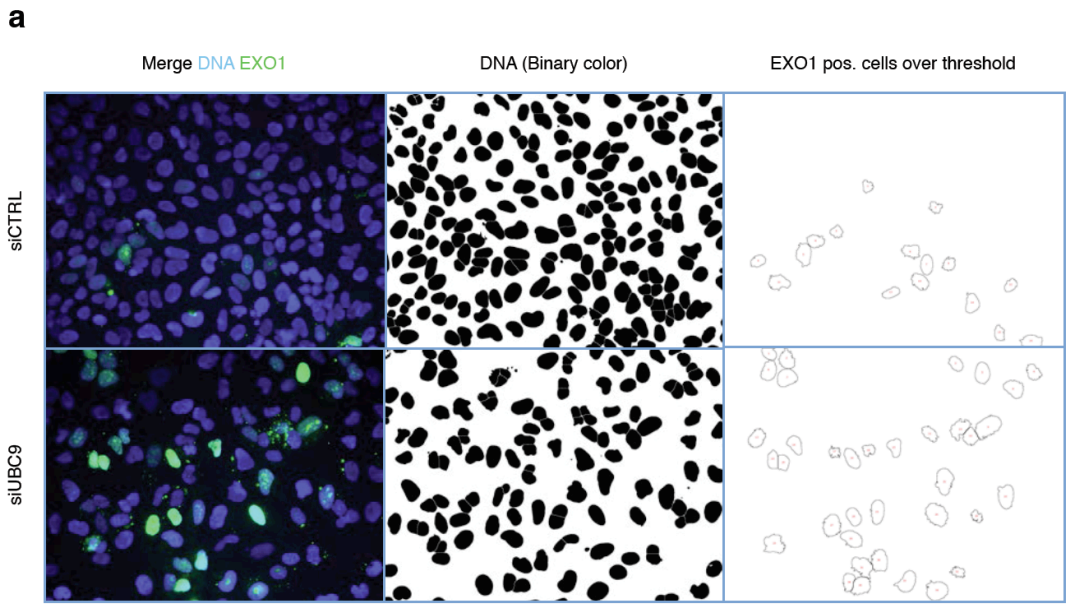

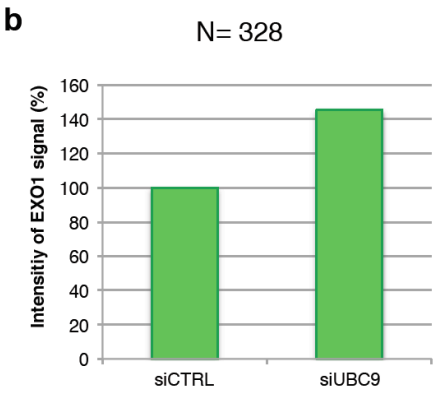

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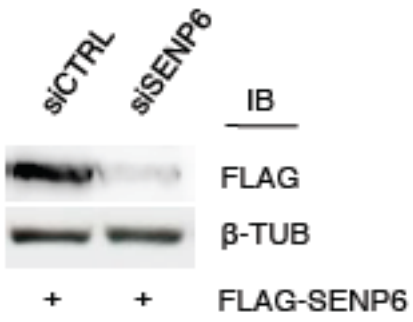

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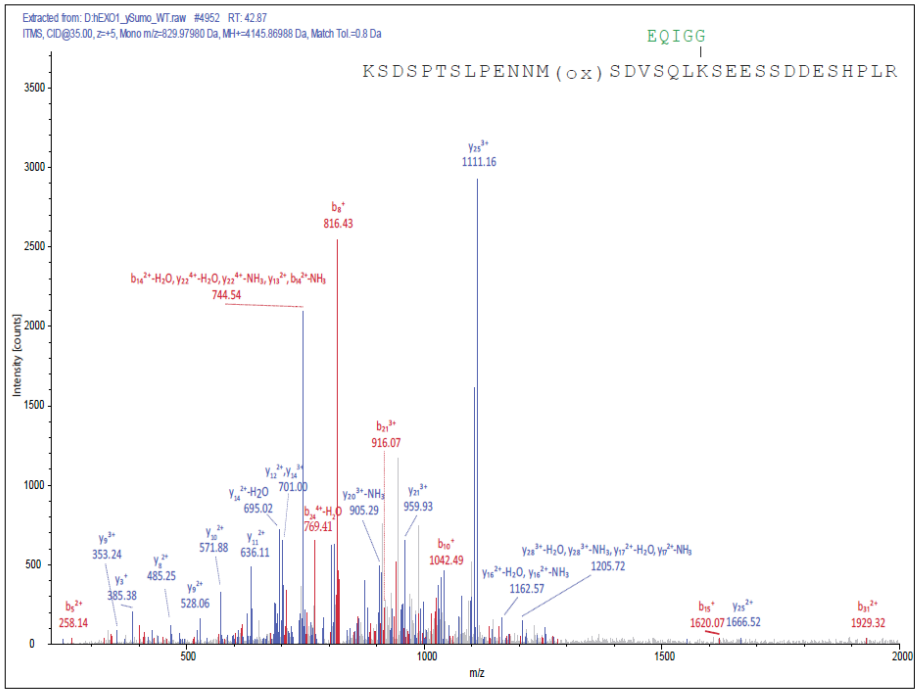

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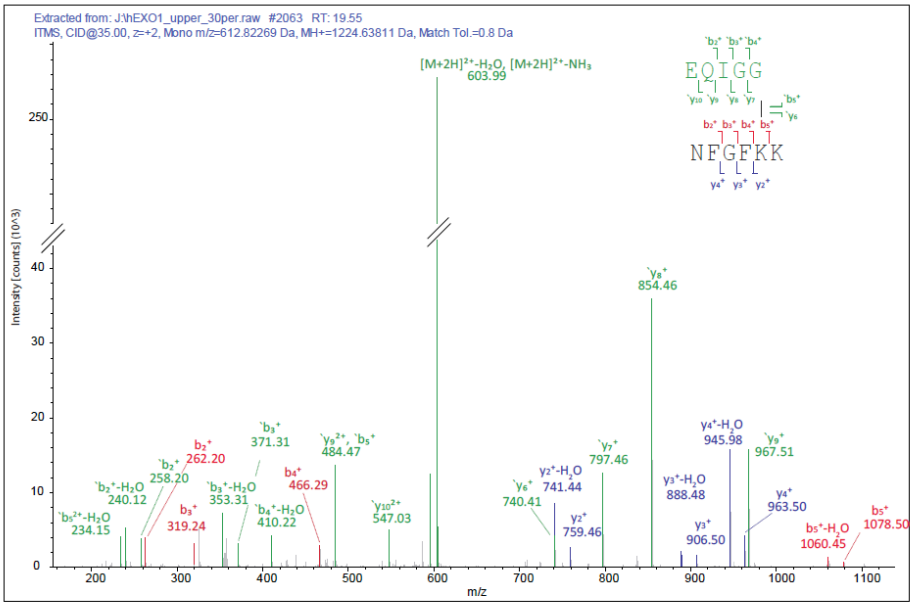

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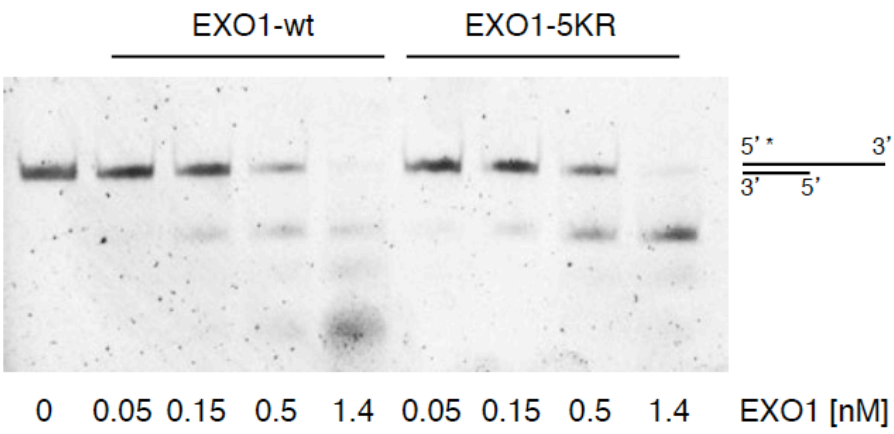

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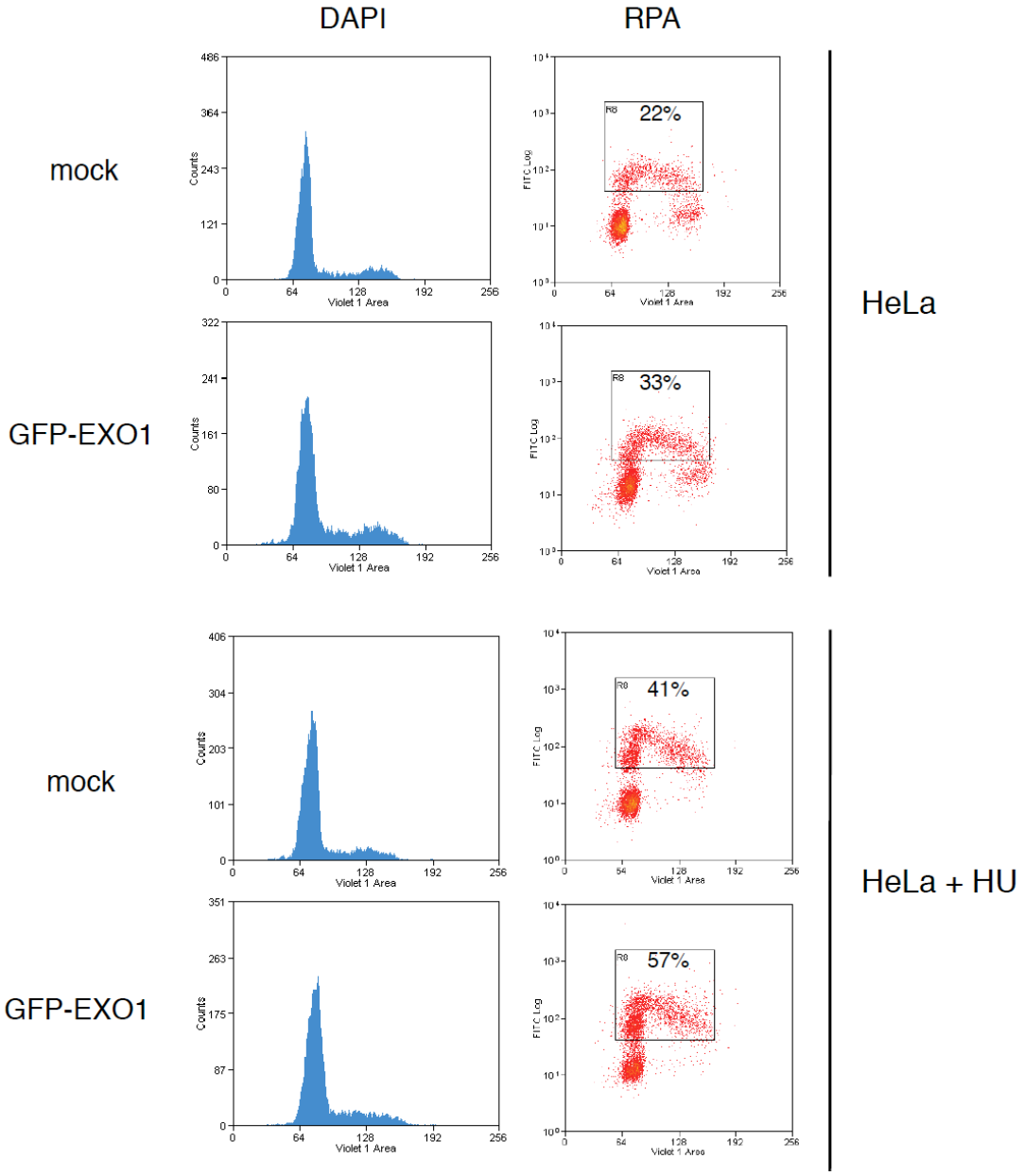

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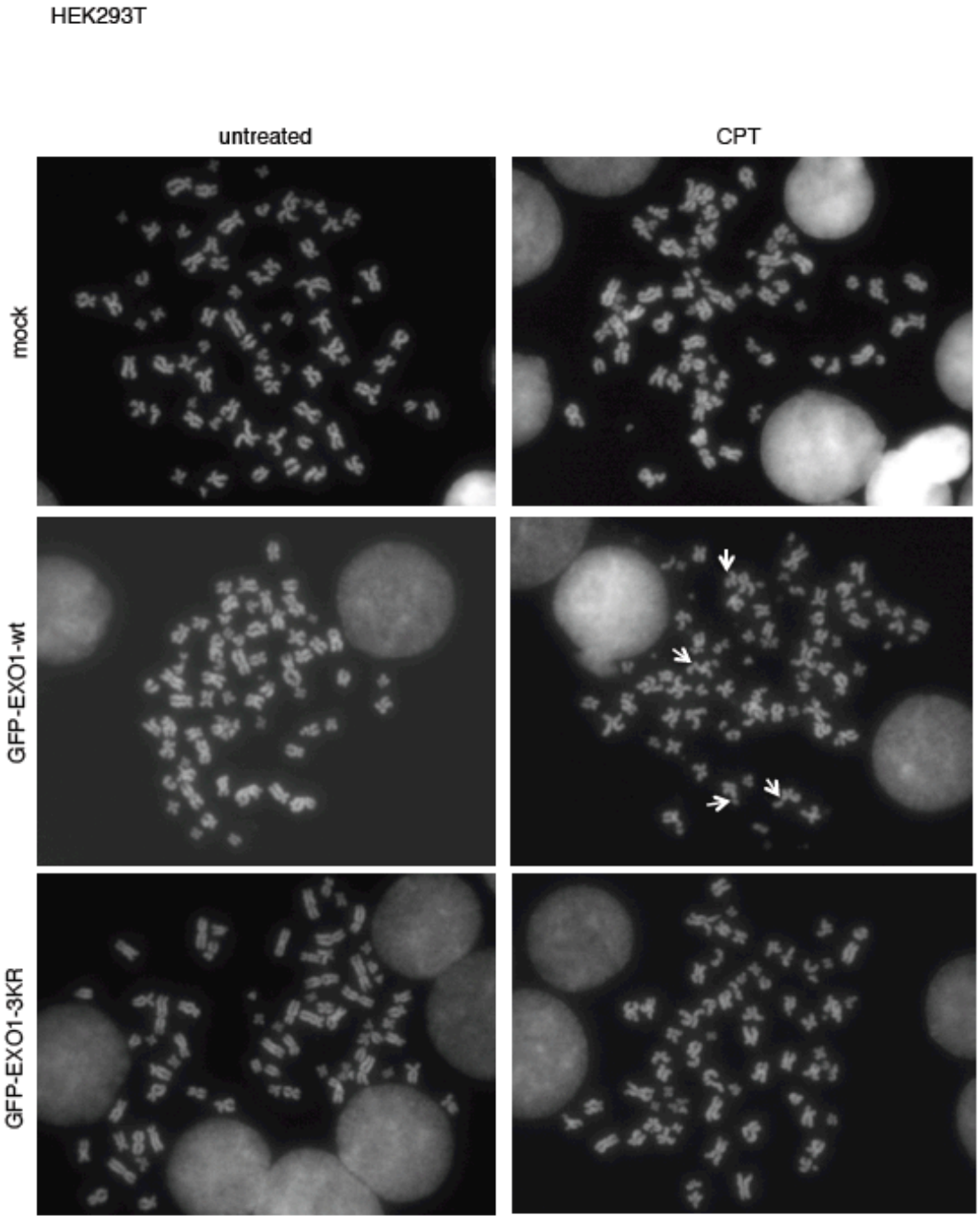

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PART II

(Unpublished data)

5. INTRODUCTION

5.1 TRIM family proteins

The RING finger motif consists of a unique linear series of conserved cysteine and histidine residues (Cys-X_2-Cys-X_{11-16}-Cys-X-His-X_2-Cys-X_2-Cys-X_{7-74}-Cys-X_2-Cys (C_3HC_4 type), where X can be any amino acid. Three-dimensional analysis of RING domains have confirmed that the RING finger motif is composed of a unique "cross-brace" arrangement with 2 zinc ions and its folding shows a small central β sheet and an α helix [1,2]. Often, the RING domain associates with cysteine-rich B-box domains followed by a predicted coiled-coil domain; a RING domain, 1 or 2 B-box domains and a coiled-coil domain at the N-terminal region of a protein is called RBCC or TRIM [3]. Studies have demonstrated that RING finger proteins have a fundamental role in growth, differentiation, transcription, signal transduction and oncogenesis [4]; more recently, proteins member of the RING family have been shown to be involved in ubiquitin-mediated degradation process.
In particular, the C_3HC_4 - type RING finger domain is found in many E3-ubiquitin ligases, such as Cbl [5], BRCA1 [6], estrogen-responsive finger protein (Efp) [7] and murine double minute 2 (Mdm2) [8]. The RING-H2 subtype in which the Cys5 is substituted by a histidine, is found in RING box protein 1 (Rbx1) and anaphase promoting complex (APC) subunit 11 (Apc11), which are components of the SCF (Skp1-Cullin-F-box) and APC E3 complexes [9]. Chu Y. and Yang X. recently published a study in which they showed that TRIM proteins represent a new class of E3-SUMO ligases and that this enzymatic activity relies on the presence of the RING domain and of an intact B-box domains [10].

5.1.1 TRIM27 as the putative EXO1 E3-SUMO ligase

TRIM27 is a member of the TRIM proteins family and it represents a clear example of the dual E3 activity features characteristic of many RING finger proteins. TRIM27 (also known as Ret finger protein or RFP) acquires oncogenic activity when it is fused to the Ret receptor tyrosine kinase [11].

TRIM27 was found to directly interact with Ubc9 and to notably stimulate Mdm2 sumoylation *in-vivo* and *in-vitro* enhancing its stability [11]. This is an example of the fact that SUMO (ubiquitin-like modifiers) molecules can compete with another post-translational modification system, namely ubiquitylation, for the attachment to Lys residues; indeed, Mdm2 stabilization upon TRIM27-dependent sumoylation is due to the consequent inhibition of ubiquitylation [12].

5.2 RESULTS

5.2.1 TRIM27 as the putative EXO1 E3-ubi ligase

A preliminary siRNA-based screening of an E2-ubiquitin conjugating enzyme library was initially performed by Western blotting analysis. The protocol for the screening was first optimized in order to be able to obtain the most pronounced exogenous EXO1 degradation upon different DNA damaging treatments (Figure 1A). In a second experiment we also tested whether the cellular stress induced by the siRNA transfection *per se* could have an effect on exogenous EXO1 protein stability (Figure 1B).

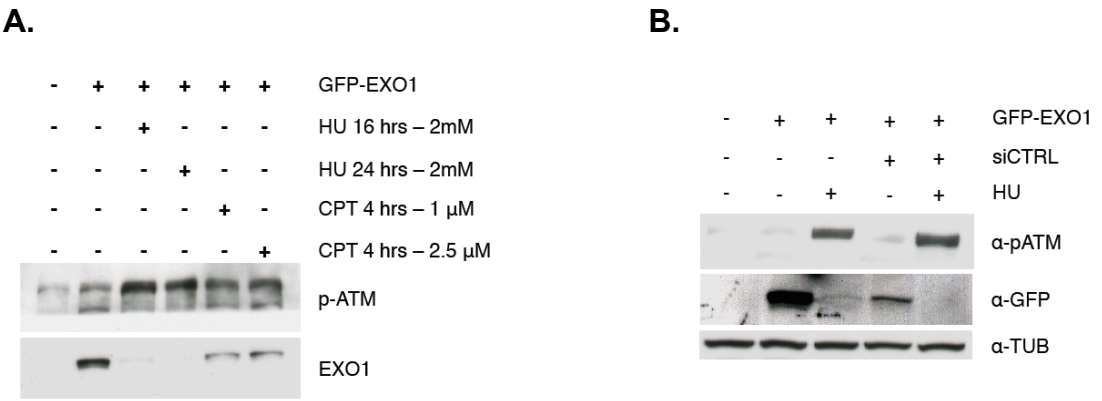


Figure 1. Protocol optimization for E2-ubiquitin conjugating enzymes screening. A) HeLa cells were transiently transfected with a GFP-EXO1 expression construct; after 8 hours cells were left untreated or treated with HU (either for 16 or 24 hours) or with CPT for 4 hours (either with 1 μM or 2.5 μM). Cells were then harvested and total cell extracts were analyzed by Western blot by using specific antibodies. **B)** HeLa cells were transfected with siCTRL for 72 hours. Transient GFP-EXO1 transfection was then performed after 48 hours post-siRNA and they were left untreated or treated with HU for 16 hours. Cells were then harvested and total cell extracts were analyzed by Western blot by using specific antibodies.

Once optimized, the protocol was applied to the screening of the library of E2 enzymes by RNAi. Few candidates, namely UBE2H, UBE2J1, UBE2L3, UBE2L6, UBE2G2, UBE2K, showing rescue of EXO1 degradation upon DNA damaging treatment, were identified (Figure 2).

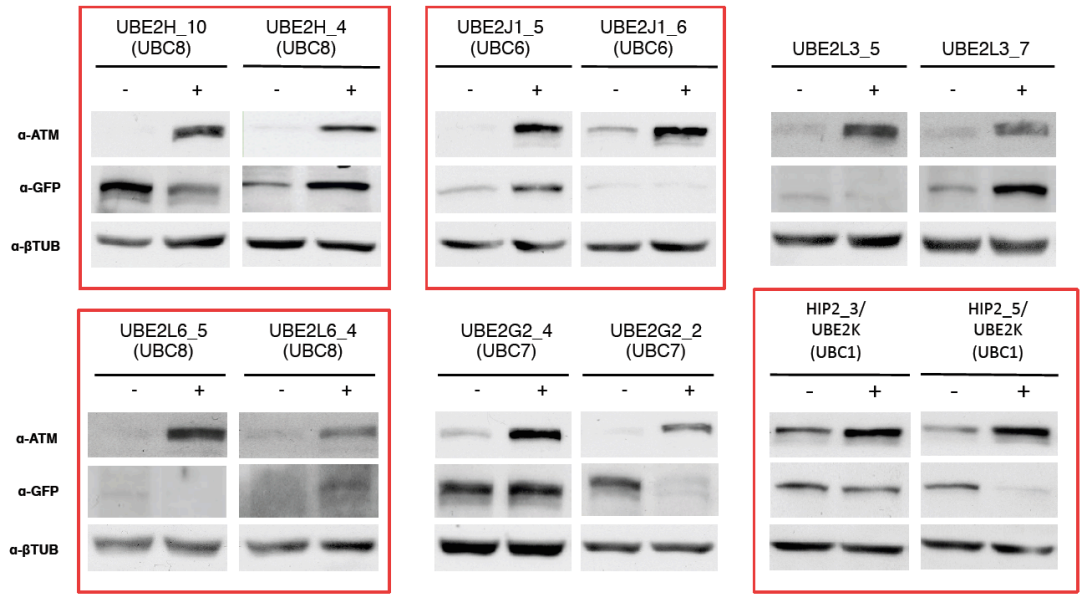


Figure 2. E2-ubiqutin conjugating enzymes' siRNA library screening.
HeLa Cells were downregulated for the specific genes coding for the E2s. For each gene, the preliminary screened library was composed of two RNA oligomers. After 48 hours post-siRNA reaction, cells were transfected with an expression plasmid encoding GFP-EXO1 and treated with HU. Whole cell lysates were analyzed by Western blotting using specific antibodies. Candidates showing abolishment of EXO1 degradation upon HU treatment are shown.

Among the six candidates identified by the screening as putative EXO1 ubiquitin-conjugating enzymes, we found that four of them have been previously reported in literature to be interacting with the RING finger protein TRIM27. Recent studies on TRIM27 revealed its dual-activity feature as an E3-SUMO and Ubi ligase, thus we decided to investigate in more detail the connection linking TRIM27 with EXO1 post-translational modifications.

In order to clarify whether TRIM27 could be involved in the DNA damage repair pathway by homologous recombination and thus, to connect it to EXO1 protein modification upon DNA damage repair, we first decided to check its regulation along the cell cycle upon Aphidicolin treatment of cells. Aphidicolin is an inhibitor of DNA polymerase α and δ causing a block of DNA replication forks that first stall and secondly collapse creating DNA double-strand breaks. 293T cells were synchronized in mitosis with the microtubule-depolymerizing agent Nocodazole and subsequently released into the cell cycle (Figure 3A). Aphidicolin was added at the time of transition into G1 (5h from the release) or during S phase (14h from the release) and TRIM27 protein level was assessed by Western blotting. Results showed that TRIM27 protein undergoes S-phase specific stabilization upon DNA damaging treatment (Figure 3B).

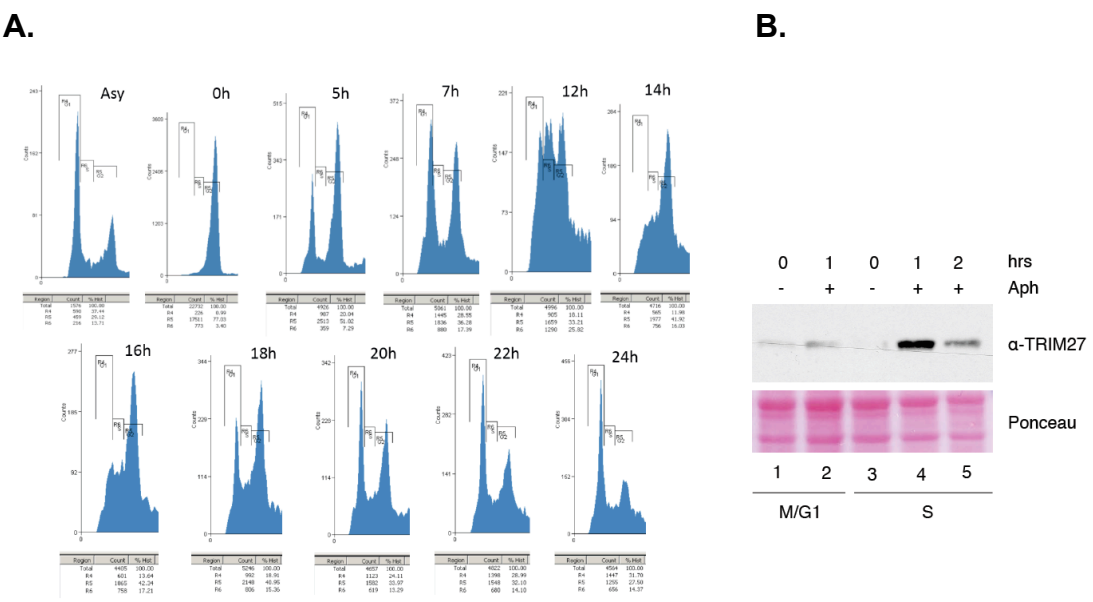


Figure 3. TRIM27 involvement in DNA damage response.
A) FACS analysis of different time points acquired from HEK 293T cells synchronized by Nocodazole (300 ng/mL) for 16 hours and released into cell cycle. B) HEK 293T cells were synchronized by treatment with nocodazole as described in A and released for 5 hours (M-G1 cells, lanes 1-2) or 14 hours (S-phase cells, lanes 3-5); at these time points, cells were left untreated (lanes 1 and 3) or treated in the presence of 5 μg/mL aphidicolin as indicated. Cell extracts were analyzed by Western blotting.

We then proceeded investigating the effects of TRIM27 downregulation on EXO1 protein stability. First, we could prove (demonstrate) TRIM27 stabilization upon another DNA damaging treatment, namely HU (Figure 4C). Secondly, we could show that depletion of TRIM27 by siRNA rescued EXO1 protein degradation upon DNA damage in GFP-EXO1 transiently- or stably-expressing U2OS cell lines (Figure 4A and 4B respectively).

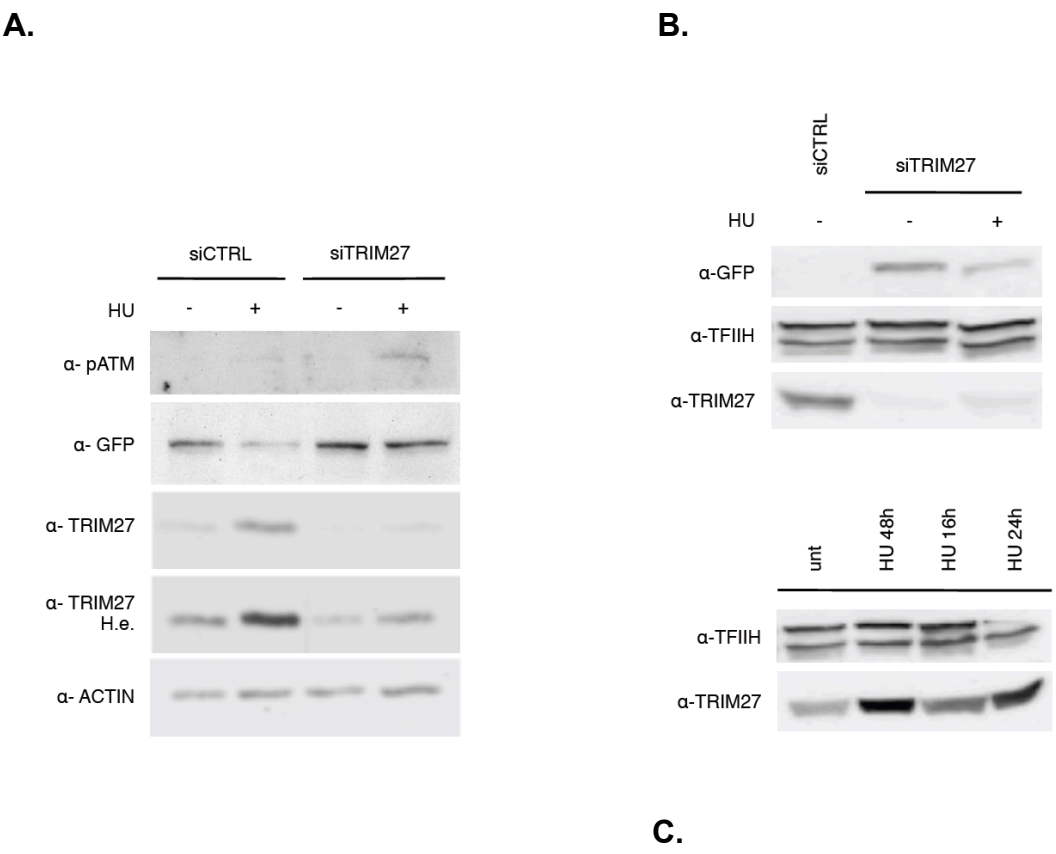


Figure 4. TRIM27 depletion stabilized GFP-EXO1 upon DNA damage.
A) U2OS cells transfected with siCTRL or siTRIM27 and transiently expressing GFP-EXO1 construct were left untreated or treated with HU for 16 hours. Cell extracts were analyzed by Western blotting. B) U2OS cells transfected with siCTRL or siTRIM27 and stably expressing GFP-EXO1 construct were left untreated or treated with HU for 16 hours. Cell extracts were analyzed by Western blotting. C) U2OS cells were left untreated or treated with HU. Cells extracts at three different time points after addition of HU to the cells were analyzed by Western blot analysis.

To confirm this last result, we performed an immunofluorescence experiment on GFP-EXO1 stable U2OS cells depleted for TRIM27. The acquired images show a pronounced increase in the GFP signal for the TRIM27 knock-down cells (Figure 5).

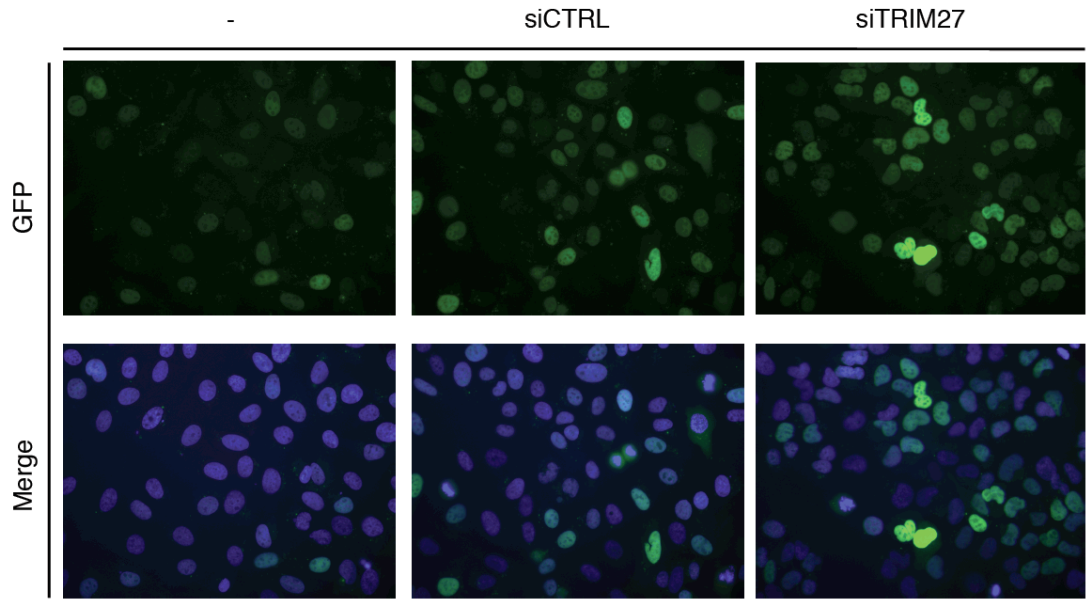


Figure 5. TRIM27 depletion stabilized GFP-EXO1 upon DNA damage.
U2OS cells stably expressing GFP-EXO1 protein were either left untreated or transfected with siCTRL or siTRIM27. IF analysis upon staining with the indicated antibody was performed.

Next, we decided to clarify the mechanism by which TRIM27 regulates GFP-EXO1 protein stability upon DNA damage. Thus, we performed a laser-microirradiation experiment on U2OS stable GFP-EXO1 cell line downregulated for TRIM27. The result showed that GFP-EXO1 could be still detected at double-strand breaks, indicating that EXO1 recruitment at damaged sites on DNA is not affected upon depletion of TRIM27.

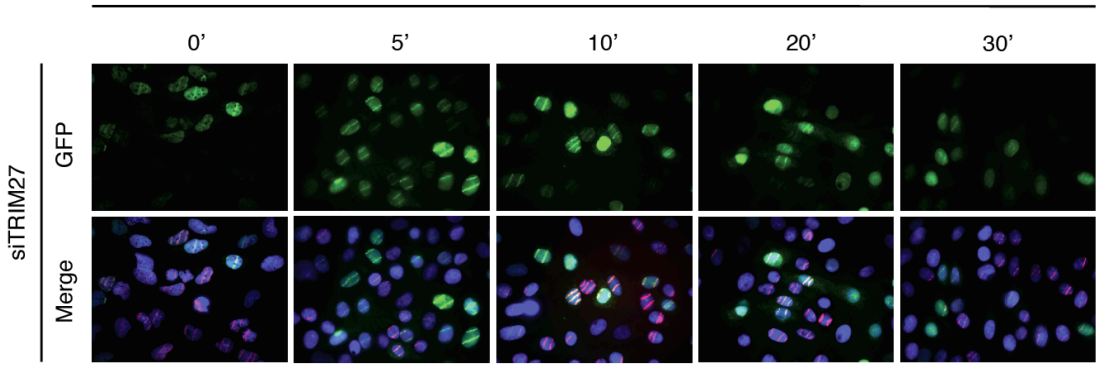
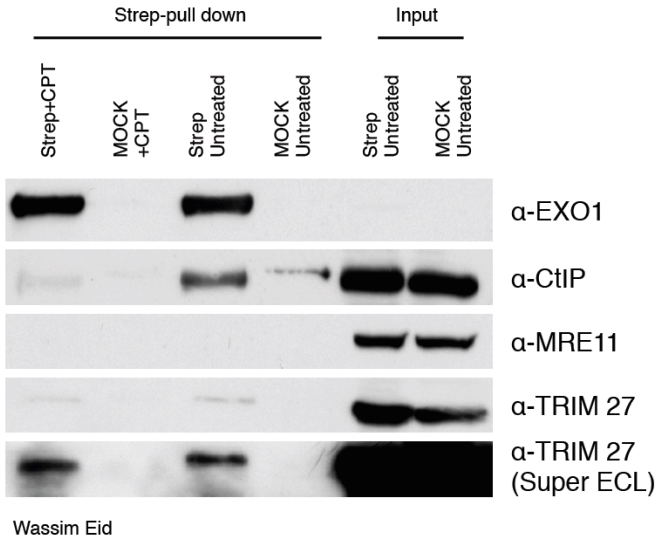


Figure 6. TRIM27 siRNA does not affect EXO1 recruitment at sites of DNA damage.
Stable U2OS GFP-EXO1 cells were depleted for TRIM27 by siRNA for 72 hours and then UVA-microirradiated. Cells were fixed at different time points upon irradiation and stained with the indicated antibody for immunofluorescence analysis.

We then assessed the interaction between TRIM27 and EXO1 by performing pull-down experiments. We were able to show the interaction between the two proteins both in-vivo and in-vitro. Moreover, the in-vivo interaction was detected in both untreated and CPT-treated cells (Figure 7).

A.



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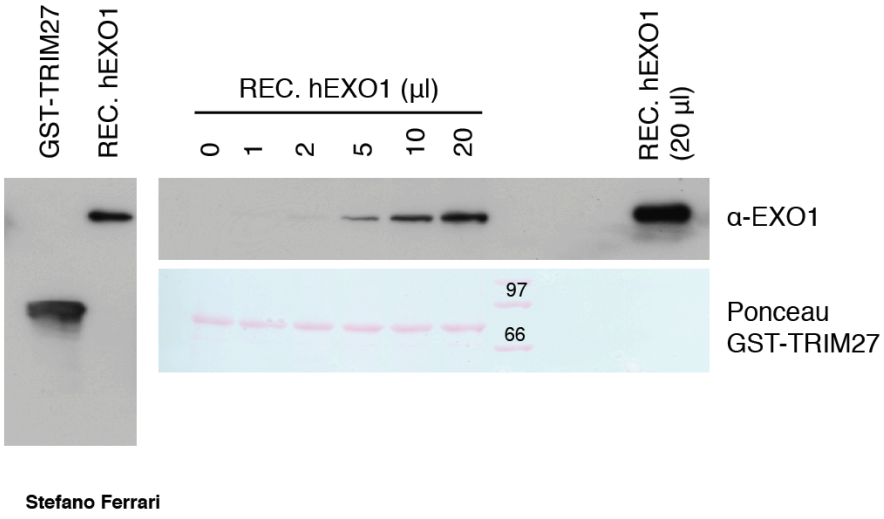


Figure 7. EXO1 interacts with TRIM27.
A) HEK 293T cells were transfected either with Strep-EXO1 or with Strep-mock constructs for 24 hours and then left untreated or treated with CPT. Cells were harvested and Strep-pull down was performed with whole cell extracts. Eluates from Strep-beads were ran on an SDS-

polyacrylamide gel and analyzed by Western blotting using the indicated antibodies. MRE11 and CtIP were used as negative and positive control, respectively, for the assay. B) In-vitro purified components (GST-TRIM27 pulled-down with glutathione beads and increasing amounts of recombinant human EXO1) were mixed. Proteins were examined by polyacrylamide gel electrophoresis. Recombinant human EXO1 protein interacting with TRIM27 was detected by using a specific antibody.

In collaboration with Prof. Martin Scheffner's laboratory at the University of Konstanz, we performed experiments testing the hypothesis that TRIM27 could be EXO1 E3-ubiquitin ligase. We first conducted preliminary *in-vitro* and *in-vivo* experiments to confirm the ability of TRIM27 to auto-ubiquitylate and thus to be active as an E3-ubiquitin ligase (Figure 8). The results showed that TRIM27 is a substrate for auto-ubiquitylation *in-vitro* and *in-vivo*. However, *in-vitro* assays including EXO1 as a substrate for ubiquitylation did not provide a clear-cut answer to our hypothesis.

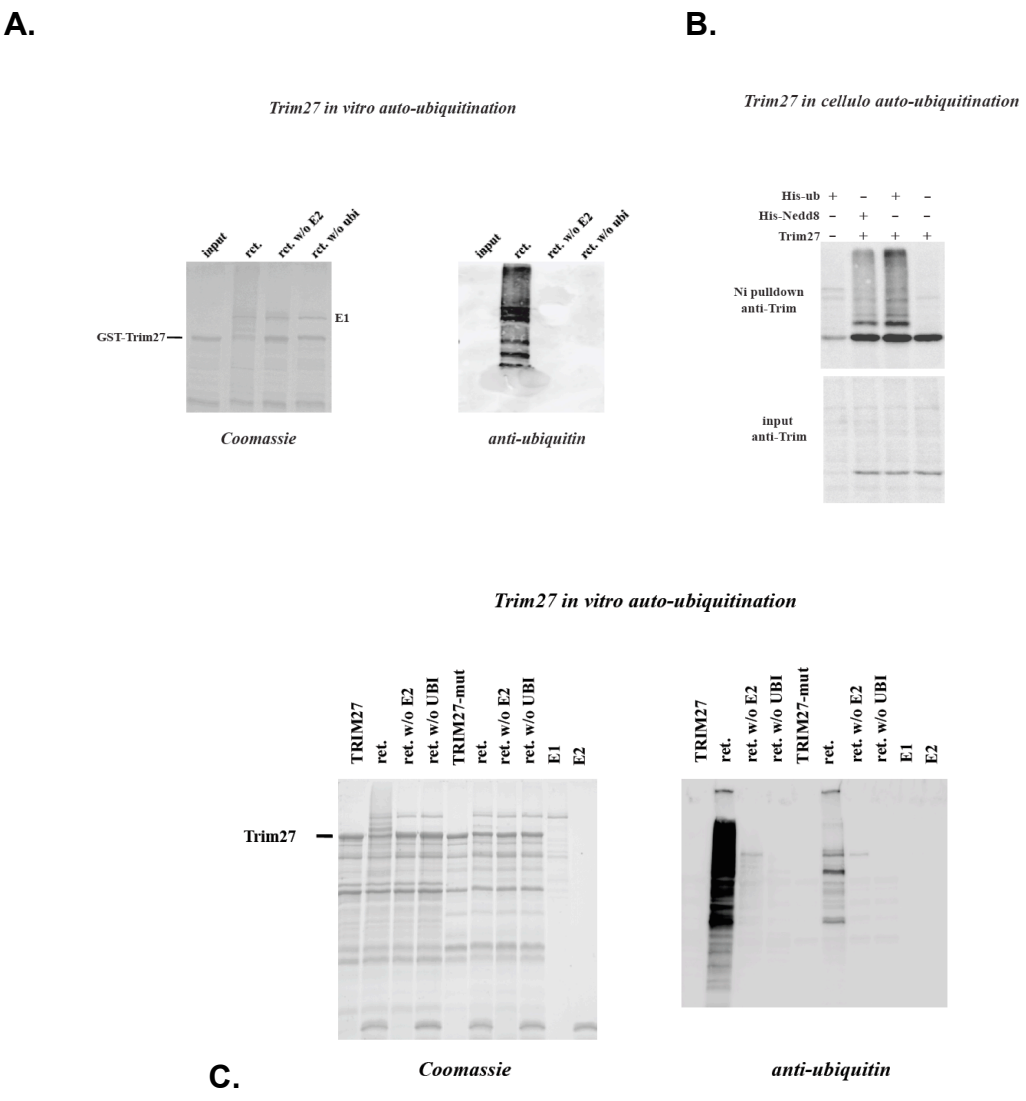
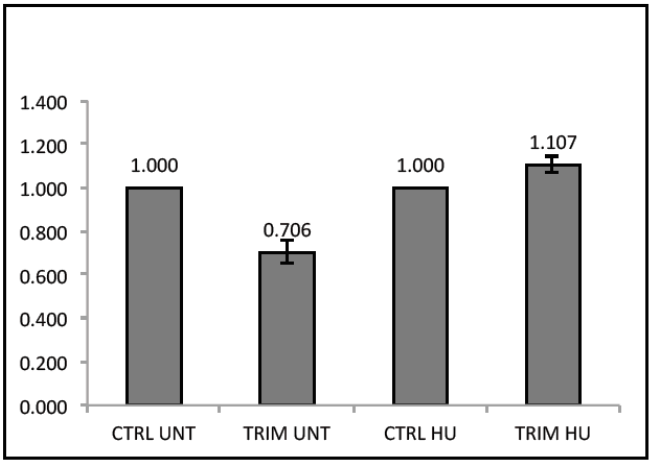


Figure 8.TRIM27 auto-ubiquitylation.
A) Coomassie staining and Western blot analysis with the indicated antibody of in-vitro ubiquitylation reactions on purified GST-TRIM27. Reactions missing either the E2-ubi conjugating enzyme Ubc5H or Ubiquitin, were used as negative controls for the assay. B) HEK 293T cells were transiently co-transfected with His-Ubi, His-NEDD8 and GFP-TRIM27 constructs. Cells were then lysed and Nichel NTA pull-down was performed on whole cell extracts. Eluates from Nichel beads were ran on an SDS-polyacrylamide gel and analyzed by Western blotting with an antibody against TRIM27. The first and the last lanes were used as negative controls for the assay. C) In-vitro auto-ubiquitylation assay on both purified TRIM27 wt and ΔRING-mutant confirmed that the intact RING-finger domain is needed for TRIM27 efficient E3-Ubi ligase activity.

Finally, since TRIM27 was reported to be a DNA binding protein acting as co-repressor of transcription (Isomura NAR 1992; Shimono JBC 2000), we decided to exclude the possibility that our observations on EXO1 protein stabilization upon TRIM27 depletion were the result of effects on transcription. Thus, we performed RT-qPCR experiments on RNA samples extracted from cells depleted for TRIM27 and we examined EXO1 mRNA levels. The assay proved that there is no significant effect on EXO1 at the transcriptional level as result of TRIM27 depletion (Figure 9).



		calibrator		test	
		CTRL UNT	CTRL HU	TRIM UNT	TRIM HU
	REP1	24.63	24.97	24.8	24.92
Ref (PBDG)	REP2	24.83	25.68	24.71	25.02
	REP3	24.65	25.56	24.7	24.72
	REP1	25.46	24.79	25.68	24.12
Target (EXO1)	REP2	25.11	24.83	25.66	24.08
	REP3	25.16	24.81	25.69	24.1
PBDG	Average	24.70	25.40	24.74	24.89
EXO1	Average	25.24	24.81	25.68	24.10

Normalize target to Ref				
2^(Ref-Target)	0.688	1.509	0.521	1.725
	CTRL UNT	CTRL HU	TRIM UNT	TRIM HU
Ratio of expression	1	1	0.757858	1.14340249

CTRL UNT	TRIM UNT	CTRL HU	TRIM HU
1.000	0.758	1.000	1.143
1.000	0.654	1.000	1.070
CTRL UNT	TRIM UNT	CTRL HU	TRIM HU
1.000	0.706	1.000	1.107

	CTRL UNT	TRIM UNT	CTRL HU	TRIM HU	2
STDEV	0	0.0734389	0	0.0519034	1.41421356
	0	0.05192914	0	0.03670124	

Figure 9. TRIM27 is not a transcriptional regulator of EXO1.
HeLa cells were transfected with siCTRL or siTRIM27 for 72 hours and then left untreated or treated with HU for the last 16 hours. Cells were harvested, total RNA was extracted and RT-qPCR analysis was performed using oligos specific for endogenous EXO1 mRNA. PBDG was used as a positive control of the assay (house-keeping gene) (data not plotted).

5.3 DISCUSSION

Ubiquitylation machinery, consisting of the three main players E1-activating, E2- conjugating and E3-ligating enzymes, represents a suitable target for interfering with post-translational modifications of its substrate proteins and thus, for de-regulating the pathways they it controls. Identifying the enzymes that attach ubiquitin molecules to EXO1 protein, thus targeting it for proteasomal degradation, will allow finding a therapeutic target the inhibition of which would result in EXO1 stabilization and DNA over-resection. This, in turn, would be the starting point to induce irreversible damage to DNA in cancer cells and triggering apoptosis as a result.

Data showed in the PART II, were aimed to investigate whether TRIM27 represents the putative E3-ubiquitin ligase for EXO1. The initially described screening of the library of E2s resulted in the identification of few candidate genes, which, once downregulated by siRNA, led to GFP-EXO1 protein stabilization. Although we decided to proceed restricting the field of E3-ligases to be screened to those reported in the literature as interacting partners of such candidates, the screening had to be repeated using a different approach due to drawbacks that conferred inconsistency to the results of the first analysis; for example, the siRNAs library had to be enlarged in the second screening in order to have at least four oligos per gene (validated and non-validated). Moreover, as described in paragraph 4, the screening was repeated in stable GFP-EXO1 U2OS cell line in order to avoid unequal GFP-EXO1 starting level between different samples due to the transient transfection protocol. Finally, we decided to perform the second screening by taking advantage of automated system for the acquisition of images of immuno-stained cells, in order to avoid the variability intrinsic to the Western blot analysis procedure.

Preliminary experiments aimed to identify the best DNA damaging treatment to be used for the screening, showed that cells treated with HU for 24 hours completely degraded GFP-EXO1, although also shorter HU treatment led to a very efficient degradation of the protein. CPT resulted, in turn, to be even stronger than HU if we consider the rate between the amount of EXO1 protein left and the length of the respective treatment. Furthermore, we could show

that the RNA interference reaction (siCTRL) itself, somehow affected EXO1 protein stability, mostly due to the cellular stress induced by the chemical reagents used in the protocol (assay, method…).

We were also able to link TRIM27 to DNA damage response pathways; indeed, results showed first that TRIM27 proteins is stabilized upon DNA damage treatment and secondly that this effect is specific for the S-phase of cell cycle, which puts TRIM27 in strong correlation with homologous recombination-based DNA damage repair pathways.

As expected, on the base of our speculation that TRIM27 is the enzyme responsible for EXO1 ubiquitylation and degradation, we could prove that depletion of TRIM27 led to exogenous EXO1 protein stabilization, both by Western blot analysis and IF.

The model established by a former post-doctoral fellow in our laboratory and based on previously published data, claims that EXO1 poly-ubiquitylation occurs once EXO1 has resected the DNA broken ends from 5' to 3' and is necessary to avoid pathological resection of DNA. Having said that, we were wondering whether EXO1 stabilization upon TRIM27 depletion could be related to the interference with the process of EXO1 recruitment at sites of damage. If this is the case, one could foresee two possible scenarios: the first is that ubiquitylation is required for EXO1 recruitment to chromatin, a condition that is no more satisfied in the absence of TRIM27; the second is that ubiquitylation is necessary to keep EXO1 on chromatin. In the absence of this modification EXO1 is released from DNA with very fast kinetics so that it is no longer detectable on chromatin. Both these explanations did not find any support from the laser-microirradiation of GFP-EXO1 U2OS cells depleted for TRIM27, since the GFP signal could still be detected at the damaged regions, indicating that EXO1 ubiquitylation occurs after its localization on DNA and that, in the absence of TRIM27, EXO1 is not ubiquitylated and thus not recognized by factors shuttling it from chromatin to the proteasome, resulting in the observed EXO1 stabilization.

Biochemical assays confirmed that TRIM27 and EXO1 constitutively interact *in-vivo* and *in-vitro*. *In-vitro* ubiquitylation assay performed on TRIM27, confirmed it to be active as E3-ubiquitin ligase being able to transfer ubiquitin molecules to itself as substrate. Moreover, the RING-domain deleted mutant

form of TRIM27 failed to undergo auto-ubiquitylation, confirming the essential feature of the RING domain for an E3 enzyme to be catalytically active. However, we could not demonstrate any direct E3-ligase activity of TRIM27 on EXO1.

Finally, we could exclude that the stabilization of EXO1 upon TRIM27 depletion was due to transcriptional regulation, since TRIM27 is known to possess also this kind of enzymatic activity.

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6. OUTLOOK

The studies reported in this thesis start shedding light on the mechanisms regulating EXO1 upon DNA damage and in particular on the post-translational modifications involved in such mechanisms.

Phosphorylation, sumoylation and ubiquitylation have been so far identified as the three main PTMs occurring on EXO1 protein and affecting its stability.
Regarding EXO1 phosphorylation, it has been previously demonstrated that among the twelve identified phosphorylation sites, three of them are induced upon hydroxyurea treatment; this data led to the hypothesis that phosphorylation is needed for the consequent EXO1 proteasome-dependent degradation, which occurs upon HU treatment. Previous reports from our laboratory also proved that EXO1 phosphorylation seems to be mostly dependent on ATR, one of the two main Kinases regulating the DNA damage response, since its depletion or inhibition leads to stabilization of EXO1 protein upon stalling of replication forks. Additionally, it was shown that upon the combined treatment of cells with HU and MG-132 proteasome inhibitor, EXO1 is phosphorylated confirming that phosphorylation precedes EXO1 degradation. S_{714} has been identified as the main DNA damage-induced phosphorylation site on EXO1, since its point mutation resulted in the most pronounced rescue of EXO1 protein upon HU treatment compared to point mutation of the other sites *(El-Shamerly, 2008)*.

In the present study, we report sumoylation, another modification found on EXO1 protein, to occur constitutively in undamaged and damaged conditions. Results, especially from experiments conducted *in-vivo*, revealed a pronounced hyper-sumoylation of EXO1 since the smeared signal detected by Western blot analysis and corresponding to sumoylated EXO1 tends to accumulate in the upper part of the gels, where the protein runs when acquiring high molecular weight due to modifications. *In-vitro* experiments mainly showed more defined and smaller modification intermediates most probably due to the reaction time length or to the components limiting amounts. We could prove that Siz1 and Siz2, yeast E3-sumo ligases, strongly

promoted EXO1 sumoyaltion *in-vitro* for both human and yeast EXO1, suggesting their involvement in the post-translational modification process. PIAS1 and PIAS4, the human homologues of Siz1 and Siz2, were also shown to affect EXO1 stability *in-vivo*, although abolishment of sumoylation upon depletion of the two ligases, supporting a direct role for these E3-ligases, is not yet available. This, together with other in-vivo experiments aimed to prove in a different way and to solidify the results proposed so far, will be performed next. Ideally, it would be also essential to show a correlation with the previously described EXO1 phosphorylation; we speculate that the three post-translational modifications mentioned here occur sequentially, as indicated by preliminary experiments described in the submitted manuscript showing abolishment of ubiquitylation upon interference with the sumoylation machinery. Thus, it will be important to investigate whether inhibition of EXO1 phosphorylation upon DNA damage, for example by using the A_{714} point mutant or in a less specific way by inhibiting ATR signal, results in abolishment or reduction of the sumoylation pattern on EXO1.

By mass spectrometry analysis of the in-vitro sumoylated human recombinant EXO1 we were able to identify some of the sumoylation sites predicted by computational tools. We created a Lys-->Arg mutant of three sumoylation sites, for *in-vitro* and mammalian expression. We could confirm an impaired sumoylation of the 3KR mutant in-vitro, although not a complete, very probably due to the fact that other lysine residues are involved. In this respect, we plan to mutate additional residues on EXO1 in order to verify whether there is a stronger effect on its stabilization and sumoylation.

Our hypothesis on the functional role of sequential PTMs occurring on EXO1 is based on the evidence published so far and on our findings that EXO1 is responsible for the second step of extended DNA resection at double-strand breaks (Lee et al., 1999); once the 5'-3' resection needed is completed, EXO1 is phosphorylated, sumoylated, ubiquitylated and degraded via proteasome. We speculate that understanding and then interfering with any of these post-translational modifications represents a clever approach aimed to create a stable and constantly enzymatically active exonuclease which does not undergo any kind of cellular regulation and which is allowed to "over-exert" its exonucleolytic activity. Over-resection of broken DNA ends could results in

the failure to repair the damage by homologous recombination, so that accumulation of un-repaired breaks as well as fusion between the extended single-strand filaments created by EXO1, will lead to progressively increased DNA damage and finally to apoptosis. Indeed, in the manuscript reported in this thesis, we show that cells over-expressing the wild type form of EXO1 to a level that likely saturates the ability of the proteasome to degrade it, leads to increased chromosome fragmentation already in unperturbed DNA conditions. This situation mimics the condition in which EXO1 over-resects DNA as consequence of inhibition of the pathways controlling its PTMs. Ideally, this hypothesis, once confirmed, could have a beneficial impact on cancer therapy; we propose, as a putative approach, the inhibition of the interaction between EXO1 and any of the factors involved in its direct modification, for example by chemical compounds competing for the interaction pocket on EXO1, associated with the most recent chemotherapeutic treatment with Olaparib and site-directed radiotherapy to selectively hit cancer cells.

In our study, this hypothesis could not be completely verified, since we observed decreased chromosomal aberrations in cells over-expressing a "stabilized" EXO1 sumoylation mutant (3KR). A possible explanation to this observation, however, is provided by other data that we present and resides in the fact that interference with the sumoylation pathway certainly causes stabilization of EXO1 protein, but also seriously affects its recruitment to chromatin, thus compromising its ability to resect DNA. Hence, we hypothesize that sumoylation of EXO1 occurs when the protein is still diffused through the nucleus; upon sumoylation EXO1 is likely targeted for recognition by a factor "X" that brings it to the damaged site on DNA, where it is then ubiquitylated and targeted for proteasomal-degradation. On the other hand, *in-vitro* DNA binding assays did not show any significant difference in the binding to DNA between the unmodified and the sumoylated form of EXO1, although a higher affinity would be expected for the latter as a confirmation of the previously provided explanation for the *in-vivo* data. This could be explained with the fact that *in-vitro,* the so called "X" factor, is not present to improve binding of sumoylated EXO1 to DNA; moreover, *in-vitro* reactions represent an artificial system where the stoichiometry of the reactions could

be very far from the *in-vivo* situation, justifying the binding to DNA of the unmodified form of EXO1.

The hypothesis that EXO1 sumoylation is needed for its recruitment to sites of DNA damage can also be exploited in cancer cell lines known to produce higher levels of EXO1 transcripts compared to normal cell lines. Indeed, we first intend to confirm that such elevated mRNA levels correlates with increased protein levels and secondly to investigate whether these cancer cells are able to silence the pathological exonucleolytic effects of the hyper expressed EXO1 by interfering with its sumoylation machinery. Possibly, components such as PIAS1 and PIAS4 are expressed at a physiological level which thus, represents a limiting factor for EXO1 sumoylation to occur. If this is the case, we would try overexpressing such components to compensate the different protein expression and test our hypothesis. In this way an additional piece of knowledge would be elucidated providing the base for future extended research on tumorigenesis and possible therapeutic approaches.

Concerning another PTMs occurring on EXO1, namely ubiquitylation, it has been shown that EXO1 polyubiquitylation can be detected upon inhibition of the proteasome with MG-132 under DNA damaging conditions *(El-Shamerly, 2005)*. In the present study, we showed that interfering with the sumoylation pathways by UBC9 down-regulation or by treating cells with Anacardic Acid, EXO1 ubiquitylation is also compromised. We plan to further investigate this sequential feature of PTMs in order to confirm that sumoylation and ubiquitylation occur directly on EXO1 and are functionally linked, excluding indirect effects of sumoylation on components of the EXO1 ubiquitylation pathway.

Unpublished data on TRIM27 were no longer considered because not all of them could be confirmed on endogenous EXO1 and await further testing. In this respect, since the identification of the E2 and E3 enzymes involved in EXO1 ubiquitylation is still missing, we would like to proceed with two different approaches: the first one consists in the repetition of the E2s-ubiquitin conjugating enzymes' siRNA library screen using a higher sensitivity protocol;

the idea is to be able to catch the finest differences between the different depletions in order to identify a small group of candidates and to be able to restrict the group of E3-ubiquitin ligases on the base of the established interaction network of this family of proteins. The second approach consists in performing mass spectrometry analysis of EXO1, examining interactors that are involved in post-translational modification pathways upon DNA damage.

7. CURRICULUM VITAE

Name: Serena

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Academic Degrees and Education

2010 - current **Ph.D**.
Cancer Network Zurich - Cancer Biology Ph.D. Program.
Life Science Zurich Graduate School - University of Zurich.
PhD thesis title 'Post-translational modifications regulating Exonuclease 1 in response to replication forks stalling and double-strand breaks' .

2009 - 2010 **Research Fellowship**
San Raffaele University - San Raffaele Scientific Institute.
Department of Molecular Biology and Functional Genomics.
Chromatin Dynamics unit.
Project title 'Real time analysis of inflammatory genes transcription in single living cells' .

2008 - 2009 **Research Fellowship**
IFOM - FIRC Institute of Molecular Oncology.
Project title 'Biochemical and biological characterization of Xtp1 protein during the cell cycle in HeLa cells' .

2005 - 2008 **Second Master's Degree in Industrial Biotechnology**
University of Milan 'Bicocca'.
IFOM - FIRC Institute of Molecular Oncology.
Thesis title 'Gene Targeting in embryonic stem cells to generate transgenic mice expressing the Bmi-1 oncoprotein in an inducible manner'.

2001 - 2005 **First Master's Degree in Biotechnology**
University of Milan 'Bicocca'.
Thesis title 'Systems of controlled drugs release based on cyclodextrins use' .

1996 - 2001 **Bachelor's Degree**
Liceo Scientifico Tecnologico 'A. Einstein'.

Working experience

November 2003 - February 2004 **Stage as undergraduate student**
I.R.A. - Istituto Ricerche Applicate S.r.l.
Research field: biological, chemical and physical evaluation of products and feedstocks for chemical-biotechnological industry.

September 2001 - October 2003/
March 2004 - October 2005 **Office employee**
Phobos S.a.s
Project field: industrial system in chemical / petrol-chemical / energy.

Technical experience

2005 - current
- Mouse embryonic stem (ES) cells culture. Mouse embryonic fibroblasts culture.
- Cre-*lox*-based system for multiple gene insertion.
- C57BL/6 and immunodeficient nude mice handling.
- Mice subcutaneous injection of ES cells.
- Histochemical and molecular analysis of teratomas.
- Generation of murine chimeras by mouse blastocysts injection with targeted ES cells.
- Gene targeting into *Rosa26* (R26) *locus* of C57BL/6-derived B6-3 murine embryonic stem cells.
- Electroporation.
- Genomic DNA isolation.
- Southern blotting.
- Transgenic knock-in mice generation for conditional gene expression.
- Cell synchronization.
- GTPases activity assay.
- Computational expertise in single-cell analysis.
- High content image-based screens (siRNA-based screens).
- Assay development for high-content screens (DNA Damage Response).
- Automated microscopy.
- PCR. Reverse-Transcription quantitative PCR.
- Site-specific mutagenesis.
- Nucleic acid purification.
- Cloning. Sequence analysis.
- Protein purification (GST-, His-tagged).
- Protein extraction from eukaryotic systems.
- SDS- PAGE gel electrophoresis.
- Western blotting.
- Direct and Indirect immunofluorescence-based analysis of proteins.

- *In-vitro* enzymatic activity and DNA binding assays.
- Pull-down. Immuno-precipitation.
- Human cell lines culture.
- RNAi.
- DNA Transfection.
- Light-microscopy. Video-microscopy.
- Site-directed DNA damage of live cells by laser microirradiation.
- Preparation of samples for Mass spectrometry analysis (Silver staining, Comassie staining).
- *In-vivo* and *in-vitro* ubiquitylation / sumoylation assays.
- Flow-cytometry samples preparation and analysis.

Awards

2013	Cancer Biology PhD Program - University of Zurich. Travel grant.
2012	UZH Forschungskredit _ Research Fellowship
2011	URPP (University Research Priority Programs) _ Research Fellowship

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Teaching experience

2013- 2014	Supervision of 1 master thesis (12 months) and 1 bachelor thesis (6 months) students.
2012	Planning, organization and supervision of University of Zurich BIO-246 block-course (2 weeks practical course for bachelor students).
2011	Supervision of University of Zurich BIO-111 course (Classical and Molecular genetics) (2 weeks practical course for bachelor students).

Attended courses

2013	Course in Supervising Students. (UZH).
2013	Course in Time Management. (UZH).
2012	EMBO practical course.

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2011	Course in Scientific Writing. (UZH).
2010	Course in Clinical Cancer research. (UZH).
2010	Course in Molecular and Cell Biology of Cancer. (UZH).
2010	Course in Science Ethics. (UZH).

Invited Seminars and Conferences

2014	Benzon Symposium N^{o} 60 - Copenhagen - Denmark. Nuclear Regulation by Ubiquitin.
2014	7th International Conference, Shanghai - China. SUMO, Ubiquitin, UBL Proteins: Implications for Human Diseases.
2012	EMBO practical course. Alghero - Italy. Ubiquitin and SUMO 2012.

Collaborations

Prof. Dr. Martin Scheffner	Laboratory of Cellular Biochemistry. Department of Biology - University of Konstanz. Germany. *In-vitro* ubiquitylation assays.
Prof. Matthias Peter	Institute of Biochemistry - Department of Biology ETHZ. Switzerland. RNAi screening of Cullins E3-ubiquitin ligases.
Prof. Lukas Pelkmans / Dr. Prisca Liberali	Institute of Molecular Life Sciences. University of Zurich. Switzerland. High content image- and siRNA-based screening of E2-ubiquitin conjugating enzymes.
Prof. Lumir Krejčí / Dr. Veronika Altmannová	National Centre for Biomolecular Research. Masaryk University - Brno. Czech Republic. *In-vitro* sumoylation assays.
Prof. Gustav Ammerer / Dr. Dorothea Anrather	Department of Biochemistry and Cell Biology. Max F. Perutz Laboratories. University of Vienna. Austria. Mass Spectrometry analysis.

List of Publications

1- Bologna S, Ferrari S.
'It takes two to tango: Ubiquitin and SUMO in the DNA damage response'
Front Genet. 2013 Jun 11;4:106. doi: 10.3389/fgene.2013.00106. eCollection 2013.

2- Bologna S, Altmannova V, Valtorta E, Koenig C, Liberali P, Anrather D, Ammerer G, Pelkmans L, Krejci L and Ferrari S*
'Sumoylation regulates EXO1-dependent resection at sites of DNA damage'
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Submitted (under review at *Nucleic Acid Research*)

3- Marchesi S, Montani F, Deflorian G, D'Antuono R, **Bologna S**, Mazzoccoli C, Di Fiore PP*.
'An adhesion-dependent checkpoint controls mitotic entry'
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Available upon request.